The Effects of Environmental Factors on Biomass and Microcystin Production by the Freshwater Cyanobacterial Genera *Microcystis* and *Anabaena*

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Cover: Valtteri N., Jussi, and assistants Jooa, Valtteri P., Kristian, Sonia, and Laura inoculating *Microcystis* 269 into different media at the Yliskylä day-care centre in Helsinki in 2006 (photo Antti Westman). The lower photo shows the cultures after three weeks of cultivation in 1) Z8 media 2) Z8 with 5‰ NaCl 3) N free Z8 4) water, where commercial nutrient fertilizer was added, and 5) tap water.

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles, which are referred to by their roman numerals in the text.

I) Chantal Vézie, Jarkko Rapala, Jaana Vaitomaa, Jussi Seitsonen, and Kaarina Sivonen (2002). Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. *Microbial Ecology* 43:443-454.

II) Jaana Vaitomaa, Jarkko Rapala, Sari Repka, Lyudmila Saari, and Kaarina Sivonen. High phosphorus and nitrogen concentrations resulted in higher biomass of a microcystin-producing *Microcystis* strain than the biomass of two nonmicrocystin-producing strains. Manuscript.

III) Jaana Vaitomaa, Anne Rantala, Katrianna Halinen, Leo Rouhiainen, Petra Tallberg, Lena Mokelke, and Kaarina Sivonen (2003). Quantitative real-time PCR for determination of *microcystin synthetase E* copy numbers for *Microcystis* and *Anabaena* in lakes. *Applied and Environmental Microbiology* 69:7289-7297.

IV) Jaana Vaitomaa, Sari Repka, Lyudmila Saari, Petra Tallberg, Jukka Horppila, and Kaarina Sivonen (2002). Aminopeptidase and phosphatase activities in basins of Lake Hiidenvesi dominated by cyanobacteria and in laboratory-grown *Anabaena. Freshwater Biology* 47:1582-1593.

THE AUTHOR'S CONTRIBUTION IN ARTICLES

I) Jaana Vaitomaa conducted the batch culture experiment of *Microcystis* GL060916 together with Dr. Chantal Vézie and contributed to the revision of the paper.

II) Jaana Vaitomaa cultivated 9 of the 12 treatments of chemostat cultures, conducted most of the analytical work to determine the biomass, microcystin and nutrient concentrations, interpreted the results, and wrote the paper.

III) Jaana Vaitomaa designed the study. She applied a real-time PCR method to quantify *microcystin synthetase E* gene copy numbers of *Microcystis* and *Anabaena* in two Finnish lakes, interpreted the results, and wrote the paper.

IV) Jaana Vaitomaa designed the study. She conducted the batch culture study of *Anabaena* 202 A1 and did most of the analyses to determine the biomass and the extracellular enzyme activities of this strain. In addition, she determined most of the phosphatase and leucine aminopeptidase activities in Lake Hiidenvesi. She analysed the data, interpreted the results and wrote the paper.

ABBREVIATIONS

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
DGGE	Denaturing gradient gel electrophoresis
ELISA	Enzyme-linked immuno sorbent assay
HPLC	High performance liquid chromatography
ICBN	International Code of Botanical Nomenclature
ICNP	International Code of Nomenclature of Prokaryotes
ISO	International Organisation for Standardisation
ITS	Internal transcribed spacer
LAP	Leucine aminopeptidase activity
LC	Liquid chromatography
kb	1000 base pairs
LD_{50}	Dose that kills 50% of treated animals
MALDI-TOF-MS	Matrix-assisted laser desorption ionisation time of flight mass spectrometry
MC-LR	Leucine and arginine in the positions of X and Z of microcystin
MC-RR	Arginine and arginine in the positions of X and Z of microcystin
mcy genes	Genes encoding the microcystin synthetase enzyme complex
MC-YR	Tyrosine and arginine in the positions of X and Z of microcystin
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
NRPS	Nonribosomal peptide synthetase
P_i	Orthophosphate-P
PC	The intergenic spacer region within the phycocyanin operon
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PPI assay	Protein phosphatase inhibition assay
QRT	Quantitative Real Time
RFLP	Restriction fragment length polymorphism
16S rRNA	Bacterial small subunit of ribosomal ribonucleic acid
WHO	World Health Organisation

ABSTRACT

Several cyanobacterial genera produce the hepatotoxins, microcystins. Microcystins are produced only in cells that have microcystin synthetase gene (*mcy*) clusters, which encode enzyme complexes involved in microcystin biosynthesis. Microcystin-producing and nonmicrocystin-producing genotypes of single cyanobacterial genus may occur simultaneously *in situ*. Previously, the effects of environmental factors on the growth and microcystin production of cyanobacteria have mainly been studied by means of isolated cyanobacterial cultures in the laboratory. Studies in the field have been difficult, owing to the lack of methods to identify and quantify the different genotypes.

In this study, genus-specific microcystin synthetase E (*mcyE*) gene primers were designed and a method to identify and quantify the *mcyE* copy numbers was developed and used *in situ*. *Microcystis* and *Anabaena mcyE* genes were observed in two Finnish lakes. *Microcystis* appeared to be the most abundant microcystin producer in Lake Tuusulanjärvi and in one basin of Lake Hiidenvesi. Because the most potent microcystin-producing genus of a lake can be identified, it will be possible in the future to design genus-targeted strategies for lake restoration.

Effects of P and N concentrations on the biomass of microcystin-producing and nonmicrocystin-producing *Microcystis* strains and an *Anabaena* strain were studied in cultures. P and N concentrations and their combined effect increased cyanobacterial biomass of all *Microcystis* strains. The biomass of microcystin-producing *Microcystis* was higher than that of nonmicrocystin-producing strains at high nutrient concentrations. The P concentration increased *Anabaena* biomass, but the effect of N concentration was statistically insignificant for growth yield, probably due to the ability of the genus to fix molecular N_2. P and N concentrations and combined nutrients caused an increase in cellular microcystin concentrations of the *Microcystis* strain cultivated in chemostat cultures.

Cyanobacteria are able to hydrolyse nutrients from organic matter through extracellular enzyme activities. Leucine aminopeptidase (LAP) activity was observed in an axenic N_2-fixing *Anabaena* strain grown in batch cultures. The P concentration caused a statistically significant increase in LAP activity, whereas the effect of N concentration was insignificant. The highest LAP activities were observed in the most eutrophic basins of Lake Hiidenvesi. LAP activity probably originated mostly from attached heterotrophic bacteria and less from cyanobacteria.

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Monet syanobakteerien eli sinilevien suvut voivat tuottaa maksamyrkkyjä, mikrokystiinejä. Myrkyntuotto on mahdollista niissä soluissa, joissa on myrkkyä tuottavia entsyymikomplekseja. Vesistöissä voi esiintyä samanaikaisesti useita eri syanobakteerisukuja sekä niiden myrkyllisiä ja myrkyttömiä muotoja. Ympäristötekijöiden vaikutuksia on tutkittu lähinnä syanobakteeriviljelmillä laboratoriossa. Tutkiminen vesistöissä on ollut vaikeaa, sillä aiemmin ei ole ollut menetelmiä, joilla maksamyrkkyä tuottaneet solut olisi voitu tunnistaa.

Tässä tutkimuksessa kehitetyllä geneettisellä menetelmällä pystytään tutkimaan maksamyrkkyjä tuottavia syanobakteereita vesistöissä, ja menetelmällä voidaan arvioida kahden tärkeimmän maksamyrkyntuottajasuvun määriä vesistöissä. Tuusulanjärvellä ja Hiidenvedellä maksamyrkyn tuottamiseen tarvittavia geenejä havaittiin *Microcystis*- ja *Anabaena*-suvun syanobakteereissa. Tuusulanjärvellä ja Hiidenveden Kiihkelyksenselällä näitä geenejä oli todennäköisesti eniten *Microcystis*-suvun syanobakteereilla. Näiden tietojen ansiosta voi olla mahdollista suunnitella sellaisia järvien kunnostusmenetelmiä, joilla voisi vähentää juuri haitallisimman syanobakteerisuvun kasvua.

Fosfori- ja typpipitoisuudet ja niiden yhteisvaikutus tehostivat *Microcystis*-suvun maksamyrkyllisten ja myrkyttömien syanobakteerien kasvua. Ravinteet edistivät erityisesti maksamyrkyllisten syanobakteerien kasvua. Fosfori- ja typpipitoisuudet ja niiden yhteisvaikutus kasvattivat kahden *Microcystis*-syanobakteerin maksamyrkkypitoisuutta. Korkea fosforipitoisuus lisäsi myös *Anabaena*-syanobakteerin kasvua, mutta typpipitoisuudella ei ollut tilastollisesti merkittävää vaikutusta sen kasvulle todennäköisesti siksi, että suku pystyy sitomaan ilmasta veteen liuennutta typpikaasua.

Syanobakteerit pystyvät irrottamaan tuottamillaan solun ulkopuolisilla entsyymeillä ravinteita orgaanisesta aineesta. *Anabaena*-puhdasviljelmässä havaittiin leusiiniaminopeptidaasiaktiivisuutta (LAP). Fosforipitoisuus kasvatti LAP-aktiivisuutta. Typpipitoisuudella ei ollut tilastollisesti merkittävää vaikutusta. Hiidenvedellä suurin LAP-aktiivisuus havaittiin järven kaikkein rehevimmissä osissa. Jos oletetaan, että syanobakteerien LAP-aktiivisuus oli Hiidenvedellä yhtä suurta kuin *Anabaena*-puhdasviljelmässä, voidaan päätellä, että Hiidenvedellä LAP-aktiivisuus oli enimmäkseen peräisin syanobakteerien yhteydessä elävistä muista bakteereista.

1. Introduction

1.1. Cyanobacteria

Cyanobacteria are photoautotrophic organisms that comprise a single phylogenetic group within the domain *Bacteria* (Castenholz 2001). They have photosystems I and II and use water as an electron donor for photosynthetic carbon dioxide reduction. Several cyanobacteria may carry out anoxygenic photosynthesis as well, using only photosystem I, if electron donors such as sulfide are present (Madigan *et al.* 2003). Cyanobacteria are thought to have existed as much as 3500 million years ago, based on documented fossil records (Schopf 2000). However, DNA-based evidence suggests that cyanobacteria appeared more recently, about 2600 million years ago (Hedges *et al*. 2001). Cell differentiation of cyanobacteria is thought to have diverged some 2100 to 2450 million years ago (Tomitani *et al*. 2006). It is widely accepted that cyanobacteria evolved oxygenic photosynthesis and changed the atmosphere of earth from anoxic to oxic, thus contributing to the development of the ozone layer (Madigan *et al.* 2003). Photosynthetic eukaryotes evolved when a eukaryotic cell engulfed and retained a cyanobacterium (Xiong *et al.* 2000, Bhattacharya *et al*. 2004).

Cyanobacteria are natural components in all kinds of aquatic habitats. In lakes, they occur in plankton, but also tightly or loosely attached to surfaces of plants, rocks, and sediments. Most cyanobacterial mass occurrences result from proliferation of planktonic cyanobacteria under favourable environmental conditions. In addition, some cyanobacteria are capable of living as terrestrial organisms on rocks and soil and are able to form symbiotic associations with plants, fungi, and animals (Whitton and Potts 2000). Cyanobacteria show wide ecological tolerance to temperature, light, and desiccation, and possess many characteristics and adaptations that explain their survival, dispersion, and success.

1.1.1. Classification

Cyanobacteria have been classified in the International Code of Nomenclature of Prokaryotes, ICNP (Oren and Tindall 2005) and in the International Code of Botanical Nomenclature, ICBN (Greuter *et al.* 2000). At the moment, efforts are being made to unify these two prevailing systems (Hoffmann 2005, Oren and Tindall 2005). Combination of morphological and molecular characteristics is another ongoing challenge for the classification of cyanobacteria (Wilmotte and Herdman 2001, Rajaniemi *et al*. 2005). Detailed information about the isolated type cultures using the form genera-cluster descriptions is presented in Bergey's Manual of Systematic Bacteriology (Castenholz 2001). Documentation of morphological characteristics of cyanobacterial isolates has been important, since some of the key morphological characteristics have been found to disappear in cultures (Gugger *et al*. 2002, Welker *et al*. 2003, Rajaniemi-Wacklin *et al*. 2006).

1.1.2. Bioactive compounds

Cyanobacteria are a rich source of bioactive compounds that may have cytotoxic, anticancer, enzyme inhibitor, antibiotic, antiviral, or antifungal properties (Moore *et al.* 1996, Namikoshi and Rinehart 1996, Burja *et al.* 2001, Sieber and Marahiel 2005). There is thus

an active search for potential new drugs amongst cyanobacterial metabolites. In general, cyanobacteria are better known for their ability to produce toxins (Sivonen and Jones 1999) that have caused adverse health effects to humans (Kuiper-Goodman *et al*. 1999). Cyanobacteria occurring in freshwater may produce hepatotoxins, microcystins, and cylindrospermopsins as well as such neurotoxins as anatoxin-a, anatoxin-a(S), and saxitoxins. Cyanobacteria may also produce endotoxins, which are inflammatory agents that may irritate any exposed tissue (Sivonen and Jones 1999). Endotoxins are components of the outer cell wall of cyanobacteria. At the time of this writing (2006), microcystins (Sivonen *et al*. 1990), anatoxin-a (Sivonen *et al*. 1989a), putative anatoxin-a(S) (Rapala *et al*. 2005b, Lepistö *et al*. 2005b), saxitoxins (Rapala *et al*. 2005b), cylindrospermopsin (Spoof *et al*. in press), and endotoxins (Rapala *et al*. 2002b) have all been detected in Finnish freshwater. In addition, cyanobacteria occurring in brackish water and in oceans produce the hepatotoxins known as nodularins as well as dermatotoxins such as aplysiatoxins and lyngbyatoxins (Sivonen and Jones 1999). In Finnish brackish waters nodularins (Sivonen *et al*. 1989b) and microcystins (Karlsson *et al*. 2005) have been observed.

1.2. Microcystins

1.2.1. Structure and synthetase genes

Microcystins are comprised of seven amino acids of Adda - D-glutamate - N-methyldehydroalanine - D-alanine - X - D-*erythro*-β-methylaspartic acid, and Z, which form a cyclic structure (Fig. 1., Sivonen and Jones 1999). The X and Z are variable L amino acids such as leucine, arginine, tyrosine, alanine, or methionine. Adda denotes 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Structural variations have been detected in all seven amino acids, especially methylation or nonmethylation of certain amino acids such as D-*erythro*-β-methylaspartic and N-methyldehydroalanine. The peptide ring forms a saddle-shaped structure, while the side chains of Adda and the

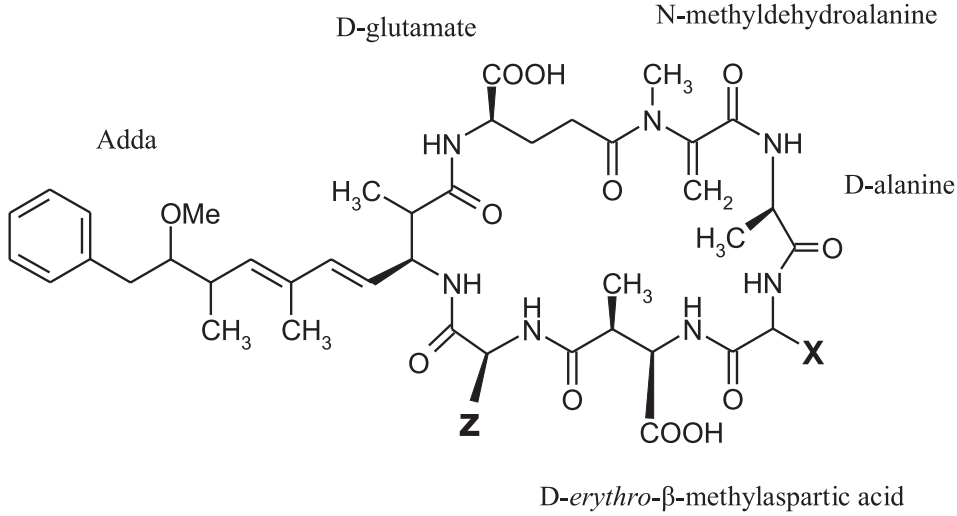

Fig. 1. Structure of microcystins in which X and Z are variable L amino acids

amino acid in position Z have been found to be very flexible, as revealed by using nuclear magnetic resonance spectroscopy (Rudolph-Böhner *et al*. 1994, Trogen *et al.* 1996, Bagu *et al*. 1997). Thus far, over 80 structural microcystin variants have been described (WHO 2004). Most of the microcystins have been structurally characterised based on material from cyanobacterial strains of the genera *Microcystis*, *Anabaena, Oscillatoria*, and *Nostoc* (Sivonen and Jones 1999).

Microcystins are synthesised nonribosomally by a polyketide synthase peptide synthetase complex and tailoring enzymes. The genes encoding enzymes of microcystin synthetase (*mcy*) have been sequenced and characterised from strains of the genera *Microcystis* (Nishizawa *et al*. 1999, Nishizawa *et al*. 2000, Tillett *et al*. 2000), *Anabaena* (Rouhiainen *et al.* 2004), and *Planktothrix* (Christiansen *et al*. 2003). In addition, the nodularin synthetase gene cluster of a *Nodularia* strain has been sequenced (Moffitt and Neilan 2001). It has been suggested that nodularin synthetase genes formed through deletions and alterations in microcystin synthetase genes (Rantala *et al*. 2004, Jungblut and Neilan 2006).

The microcystin synthetase gene cluster is comprised of 10 genes in *Microcystis* and *Anabaena*, and 9 genes in *Planktothrix*. Eight of these genes (*mcyA, mcyB, mcyC, mcyD, mcyE, mcyG, mcyH*, and *mcyJ*) are very similar, although the arrangement of some of the genes is different in the strains of the four genera (Börner and Dittmann 2005). In the *mcy* gene cluster of *Planktothrix*, the genes *mcyF* and *mcyI* are absent and an additional gene, *mcyT*, is present. The sizes of the *mcy* gene clusters of the three genera are 53 - 55 kb.

The NRPS synthetases and PKS synthases involved in the biosynthesis of microcystin have a modular structure, each module catalysing the incorporation of microcystin precursors. These modules may include modifying activities. The integrated NRPS and PKS modules in the microcystin biosynthesis are coded by *mcyG* and *mcyE*. The gene *mcyD* is coding for PKS modules. At first the McyG, McyD, and McyE catalyse the formation of the Adda - D-glutamic acid structure of the microcystin. The first module of McyG is responsible for the activation of the putative starter molecule, phenyl acetate. McyD has two polyketide synthase modules, which catalyse the incorporation of the two-carbon units in the Adda biosynthesis. McyJ is responsible for the O methylation of the Adda (Christiansen *et al*. 2003). McyE catalyses the incorporation of the last two-carbon unit of the Adda and activation and -condensation of the D-glutamate. After the formation of the Adda - D-glutamic acid structure of the microcystin, the modules coded by the NRPS genes *mcyA*, *mcyB*, and *mcyC* catalyse the incorporation and modification of the five amino acid residues in the microcystin, N-methyldehydroalanine - D-alanine - X - D-*erythro*-β-methylaspartic acid - Z presented in Fig. 1. The release and cyclisation of the microcystin is catalysed by thioesterase activity coded by the gene *mcyC*. Based on a phylogenetic analysis, the gene *mcyH* has been thought to encode a component of the ABC transporter system, which may have the function of exporting microcystin (Pearson *et al*. 2004). The numerous variants of microcystins are partly due to the rather moderate substrate specificity of some of the adenylation domains and partly due to the differences in modifying activities,

such as epimerisation and N-methylation activities found in some modules.

1.2.2. Detection and analysis

Cyanobacterial cells can be broken by methods such as a probe sonicator (Rapala *et al.* 2002a) and shaken with silica beads in a beadbeater (Wiedner *et al.* 2003) in order to release cellular microcystins, since in general, most of the microcystins are within the cells. Microcystins may be extracted into various solvents such as aqueous methanol and acetic acid (Rapala *et al.* 2002a, Barco *et al.* 2005, Meriluoto and Codd 2005). Microcystins can be concentrated and impurities removed from samples by using reversed-solid-phase extraction cartridges such as hydrophilic lipophilic balanced Oasis (Waters Corp.) or C_{18} silica (Rapala *et al.* 2002a). Brine shrimp (*Artemia salina*) and mouse bioassays (Harada *et al.* 1999, McElhiney and Lawton 2005) have been used for preliminary screening of the toxicity of environmental samples. These bioassays have some shortcomings. If samples contain several different cyanobacterial toxins as well as other toxic compounds, the faster-acting toxins such as neurotoxins may mask the slower-acting toxins such as microcystins in the bioassays.

Microcystin concentrations can be assessed and determined with biochemical, immunological, and analytical methods (Harada *et al.* 1999, McElhiney and Lawton 2005, Meriluoto and Codd 2005). The protein phosphatase inhibition (PPI) assay can be used to assess the toxicity of samples (An and Carmichael 1994). Heating the sample at 80 ºC for 15 minutes has been used to decrease the activity of heat unstable inhibiting or activating compounds of the protein phosphatases (Rapala *et al.* 2002a). The method is sensitive and has been used to quantify toxicity of microcystins in drinking water (Lambert *et al.* 1994).

Microcystins can be detected and total concentration determined with enzyme-linked immuno-sorbent assay, ELISA, which is an immunological detection method based on either polyclonal (Chu *et al.* 1989) or monoclonal antibodies (Kfir *et al.* 1986, Chu *et al.* 1990). The microcystin concentrations are obtained according to standards based on microcystin-LR in commercial ELISA microcystin plate kits. However, the cross-reaction of different microcystin variants with antibodies varies and thus may cause variation of the concentration expressed as microcystin-LR equivalents. The manufacturers provide coefficients to correct the concentrations of some variants. Commercial ELISA methods are sensitive, and generally no concentration of microcystins with reversed-solid-phase extraction cartridges is needed for environmental samples (Rapala *et al.* 2002a).

High performance liquid chromatography (HPLC) combined with UV detection (Harada *et al.* 1999, Meriluoto and Codd 2005) has been the most common method of identifying and quantifying microcystins (Table 1; Sivonen and Jones 1999). The International Organisation for Standardisation, ISO, has published a standard method for the extraction, separation, and detection of microcystin-RR, microcystin-YR, and microcystin-LR using reverse phase HPLC followed by UV detection (ISO 20179 2005). In general, a reverse-phase C_{18} silica columns together with varying proportions of ammonium acetate and acetonitrile or trifluoroacetic acid and acetonitrile as a mobile phase have been used to separate the microcystins (Harada *et al.* 1999, Meriluoto and Codd 2005). The tentative

identification of microcystins is based on retention times measured at 238 nm. Characteristic UV absorption spectra of microcystins may aid in identification, if the system has a photodiode array UV detector. Since only few variants of microcystins are commercially available, identification and quantification of other variants is problematic.

With combined liquid chromatography-mass spectrometry (LC-MS), microcystin variants can be separated and identified. With MS identification, microcystins can be identified more precisely than with UV-detection, although some variants have identical molecular weights. In those cases, model compounds would be needed for identification. Microcystin variants may be identified also based on fragment ions. ISO has started to develop a standard method to detect microcystins with LC-MS. A matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS) method has been developed to identify microcystins from small sample volumes (Erhardt *et al.* 1997). However, this method is not quantitative. Microcystins have also been detected and concentrations measured with other types of methods, such as thin-layer chromatography and capillary electrophoresis (Harada *et al.* 1999, McElhiney and Lawton 2005, Meriluoto and Codd 2005).

1.2.3. Exposure routes and toxicity

Animal poisonings and human illness attributed to cyanobacterial toxins have been reported in many countries (Kuiper-Goodman *et al*. 1999). *Microcystis* is the most common genus implicated in intoxications. In Brazil, at least 55 patients died after receiving a microcystin-containing dialysis medium in a haemodialysis center (Jochimsen *et al.* 1998). In general, microcystins may enter the body through oral consumption or inhalation of contaminated material. Thus, microcystins pose a health hazard to humans through contaminated drinking water and recreational exposure to cyanobacteria (Falconer *et al*. 1999). Microcystins constitute a health risk, particularly when they appear in raw water sources of poorly equipped drinking water plants. Dissolved microcystins in surface waters have passed through conventional water treatment plants (Jochimsen *et al.* 1998, Lahti *et al.* 2001). In Finland, microcystin concentrations detected in treated drinking water were low (Lahti *et al.* 2001) and below 1 μg l^{-1}, which is proposed by the WHO (2004) as a guideline for maximum microcystin-LR concentration in drinking water. In China, higher incidences of liver cancer were observed among people drinking pond and ditch water than among people using deep well water. The higher incidences of cancer were thought to be attributable to cyanobacterial toxins occurring in the pond and ditch water (Yu 1995). In Finland, people have suffered adverse health effects after throwing cyanobacteria-containing water that had been used also for washing onto the sauna stove (Salmela *et al*. 2001). Although their subsequent symptoms were not necessarily from the microcystins, it is possible that heat-resistant microcystins were released into the water vapour due to the rupture of cyanobacterial cells by the heat of the sauna stove and subsequently entered the body via inhalation. The intranasal administration of microcystin has shown higher toxicity than gastrointestinal administration in mice (Fitzgeorge *et al.* 1994). Cyanobacteria-containing food supplements may also form a risk, since

Table 1. The effects of P and N concentrations and growth rate on toxicity or cellular microcystin concentration (MC) of *Microcystis* strains cultivated in cultures.

Studied nutrient concentrations	Strain	Effects on toxicity or cellular microcystins concentration	Toxin(s) method	Other growth conditions	Reference
Phosphorus					
0.7-14 mg P l^{-1}	M228	0.8-fold lower toxicity at 14 mg l^{-1} than at 0.7 mg l^{-1}	Mouse bioassay	Batch cultures, Temperature, 25 ºC Light, 30 µmol m^{-2} s^{-1}	Watanabe and Oishi 1985
Deplete, 7 mg P l^{-1}	7813	No differences in toxicity	Mouse bioassay	Batch cultures, Temperature, 25 ºC Light, 15 µmol m^{-2} s^{-1}	Codd and Poon 1988
0.45, 4.45 mg P l^{-1}	CYA 228/1	2.3-fold higher MC at 4.45 mg l^{-1} than at 0.45 mg l^{-1}	HPLC	Chemostat, 0.013 h^{-1} Temperature, 20 ºC Light, 75 µmol m^{-2} s^{-1}	Utkilen and Gjølme 1995
1.4, 14 mg P l^{-1}	*Microcystis viridis*	0.6-fold lower MC at 14 mg l^{-1} than at 1.4 mg l^{-1} on day 5	HPLC	Batch cultures, 7 d Light, 80 µmol m^{-2} s^{-1}	Song *et al.* 1998
0.02-2.21 mg P l^{-1}	UTEX2388	P had no effects on MC	HPLC	Batch cultures, 7 d Temperature, 30 ºC Light, 150 µmol m^{-2} s^{-1}	Lee *et al.* 2000
0.186 mg P l^{-1}	UTEX2388	0.5-fold lower MC at growth rate 0.8 d^{-1} than at 0.1 d^{-1}	HPLC	Chemostat, Growth rates, 0.1-0.8 d^{-1} Temperature, 28 ºC Light, 160 µmol m^{-2} s^{-1}	Oh *et al*. 2000
M IV/2 medium and P limitation	HUB 5-2-4	1.4-fold higher MC-RR in M IV/2 medium than under P limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001
M IV/2 medium and P limitation	W334	1.2-fold higher MC-LR in M IV/2 medium than under P limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001
M IV/2 medium and P limitation	W368	0.5-fold lower MC-YR in M IV/2 medium than under P limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001

Table 1 continuing

Nitrogen					
1.3-26 mg N l^{-1}	M228	0.7-fold lower toxicity at 26 mg l^{-1} than at 1.3 mg l^{-1}	Mouse bioassay	Batch cultures, 15 d Temperature, 25 ºC Light, 30 µmol m^{-2} s^{-1}	Watanabe and Oishi 1985
Deplete, 12 mg N l^{-1}	7813	0.2-fold lower toxicity at 12 mg N l^{-1} than under N removal	Mouse bioassay	Batch cultures, Temperature, 25 ºC Light, 15 µmol m^{-2} s^{-1}	Codd and Poon 1988
4.9-81.2 mg N l^{-1}	CYA 228/1	3-fold higher MC at 81.2 mg l^{-1} than at 4.9 mg l^{-1}	HPLC	Chemostat, 0.013 h^{-1} Temperature 20 ºC Light, 40 µmol m^{-2} s^{-1}	Utkilen and Gjølme 1995
0.165-16.5 mg N l^{-1}	MASH01	2.8-fold higher MC at 16.5 than at 0.165 mg l^{-1} on day 17	HPLC	Batch cultures, 24 d Temperature, 20 ºC Light, 38 µmol m^{-2} s^{-1}	Orr and Jones 1998
0.12-15.5 mg N l^{-1}	MASH01-A19	3.5-fold higher MC at 15.5 than at 0.12 mg l^{-1} on day 7	HPLC	Batch cultures, 21 d Temperature, 20 ºC Light, 38 µmol m^{-2} s^{-1}	Orr and Jones 1998
2.6, 26 mg N l^{-1}	*Microcystis viridis*	1.4-fold higher MC at 26 mg l^{-1} than at 2.6 mg l^{-1} on day 5	HPLC	Batch cultures, 7 d Light, 80 µmol m^{-2} s^{-1}	Song *et al.* 1998
0.09-9.1 mg N l^{-1}	UTEX2388	1.3-fold higher MC at 1.5 mg l^{-1} than at 0.09 mg l^{-1}. 0.9-fold lower MC at 9.1 mg l^{-1} than at 0.09 mg l^{-1}.	HPLC	Batch cultures, 7 d Temperature, 30 ºC Light, 150 µmol m^{-2} s^{-1}	Lee *et al.* 2000
M IV/2 medium and N limitation	HUB 5-2-4	1.3-fold higher MC-RR in M IV/2 medium than under N limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001
M IV/2 medium and N limitation	W334	2.0-fold higher MC-LR in M IV/2 medium than under N limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001
M IV/2 medium and N limitation	W368	0.5-fold lower MC-YR in M IV/2 medium than under N limitation	HPLC	Semicontinuous cultures Temperature, 20 ºC Light, 110 µmol m^{-2} s^{-1} 12 h	Hesse and Kohl 2001
2.8 mg N l^{-1} (N deficiency)	MASH01-A19	6-fold higher MC at growth rate 1.08 d^{-1} than at 0.1 d^{-1}	HPLC	Chemostat, Growth rates, 0.1-1.08 d^{-1} Temperature, 26 ºC Light, 40 µmol m^{-2} s^{-1}	Long *et al.* 2001

microcystins and *Microcystis mcy* genes have been detected in commercial 'health food' products (Saker *et al.* 2005b).

Both microcystins and nodularins inhibit the eukaryotic serine- and threonine-specific protein phosphatase 1 and 2A (Honkanen *et al.* 1990, MacKintosh *et al.* 1990, Yoshizawa *et al.* 1990), which are essential in regulation of the eukaryotic cell cycle and in cellular metabolism (Wishart and Guan 2005). Microcystins act also as tumour promoters (Falconer and Buckley 1989, Nishiwaki-Matsushima *et al.* 1992, Ohta *et al.* 1992). In addition, microcystin-LR has been proposed to have mutagenic effects (Zhan *et al.* 2004). In mammals, microcystins are transported, particularly through bile acid-type transporters, from the ileum into the bloodstream and to hepatocytes of liver (Kuiper-Goodman *et al.* 1999). In case of acute microcystin exposure, severe liver damage results from disruption of the liver tissue structure. The inhibition of the protein phosphatases by microcystins results in hyperphosphorylation of the proteins associated with the cytoskeleton leading to cell distortion (Eriksson *et al.* 1992, Carmichael 1994). In acute microcystin poisonings, death is due to liver haemorrhage or liver failure.

Different microcystin variants exhibit different hepatotoxicities. In general, the intraperitoneal LD_{50} values of mice have varied between 50 – 300 μg kg^{-1} of body weight (Sivonen and Jones 1999). The linear variants have been less toxic than the cyclic microcystins (Choi *et al.* 1993). The few known nontoxic variants are the 6Z-Adda isomers (Harada *et al.* 1990) and the microcystins, in which the α-carboxyl group of the D-glutamate has been esterified (Namikoshi *et al.* 1992). Determination of the crystal structure of microcystin-LR bound to protein phosphatase 1 showed that binding was due to the interactions with three regions of the phosphatase (Goldberg *et al.* 1995). These were interactions with the two catalytic metals of the active site by binding two water molecules through the α-carboxyl group of the D-glutamate, the β_{12}-β_{13} loop region, and the hydrophobic groove of the phosphatase, which the Adda amino acid occupies.

1.2.4. Genera with microcystin-producing strains

Microcystin production has been reported in planktonic, benthic, and terrestrial strains of the cyanobacterial genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Anabaenopsis* (Sivonen and Jones 1999, Oksanen *et al.* 2004), *Oscillatoria* (Brittain *et al.* 2000, Oudra *et al.* 2002), *Radiocystis* (dos S. Vieira *et al.* 2003), *Arthrospira* (Ballot *et al.* 2004, Ballot *et al.* 2005), *Phormidium* (Jungblut and Neilan 2006), *Pseudanabaena* (Oudra *et al.* 2002), *Synechocystis* (Oudra *et al.* 2002), and picoplanktonic cyanobacteria (Domingos *et al.* 1999). The microcystin spectrum varies from strain to strain (Sivonen and Jones 1999). Thus, some microcystin-producing strains are more toxic than others. The microcystin production of isolated strains has been continuous, although such growth conditions as nutrient concentrations and light have had effects on cellular microcystin concentrations (Table 1; Sivonen and Jones 1999) and on the proportions of the microcystin variants produced (Rapala *et al.* 1997, Rapala and Sivonen 1998, Böttcher *et al.* 2001, Tonk *et al.* 2005). *Anabaena* and *Microcystis* are the most common mass occurrence-forming genera of cyanobacteria in Finnish freshwater (Sivonen *et al.* 1990, Lepistö *et al.* 2005b).

1.2.4.1. The genus *Microcystis*

Microcystis strains isolated in different parts of the world have been found to produce a wide variety of microcystins (Sivonen and Jones 1999). Within the studied strains of this genus, no neurotoxin or liver-targeting toxins other than microcystins has been observed. It has been believed that *Microcystis* is a freshwater cyanobacterial genus, but in a Turkish brackish-water lagoon (salinity 7 ‰), microcystin-producing *Microcystis* has formed mass occurrences (Albay *et al*. 2005). Some *Microcystis* species classified according to the rules of the ICBN have been related to a higher probability of certain microcystin variants and to other oligopeptide production than some other *Microcystis* species (Fastner *et al.* 2001, Kurmayer *et al*. 2002, Via-Ordorika *et al*. 2004, Welker *et al*. 2004). However, assigning the microcystin production to certain *Microcystis* species is difficult, since the classification of the species is unclear. For example, Otsuka *et al.* (2001) have proposed unification of the most common *Microcystis* species due to their high DNA-DNA reassociation values. *Microcystis* cells are spherical and grow as single cells in most liquid cultures, whereas *in situ*, the cells generally form tight or loose colonial aggregates, which are sometimes covered by thick mucilage.

1.2.4.2. The genus *Anabaena*

Anabaena strains have been found to produce saxitoxins, anatoxin-a, anatoxin-a (S) in addition to numerous microcystin variants (Sivonen and Jones 1999), and cylindrospermopsin (Schembri *et al*. 2001, Spoof *et al*. in press). At least one *Anabaena* strain has been reported to produce simultaneously several microcystins and anatoxin-a(S) (Sivonen and Jones 1999). Although the genus *Aphanizomenon* has morphological characteristics different from *Anabaena in situ*, phylogenetic studies have shown that these genera are intermixed (Lyra *et al*. 1997, 2001, Gugger *et al*. 2002, Rajaniemi *et al*. 2005). However, no microcystin-producing *Aphanizomenon* strains have been found. *Anabaena* grows as filaments, and the cells are spherical or cylindrical. In liquid cultures, the filaments are separate, whereas *in situ*, the filaments form tight or loose colonial aggregates, and the filaments may be covered by mucilage.

1.2.5. Identification of microcystin-producing genera *in situ*

Microcystin-producing and nonmicrocystin-producing strains within a single cyanobacterial genus cannot be differentiated with any direct light microscopical method. Isolation, cultivation, and toxin analysis of strains of cyanobacteria were long the only means of verifying that some cyanobacteria were able to produce toxins. In recent years, new techniques for identification of microcystin-producing cyanobacteria have been developed.

The *mcy* genes of the genera *Microcystis*, *Anabaena,* and *Planktothrix* (Börner and Dittmann 2005) have been used to design identification and detection methods for the potentially microcystin-producing genera. The *mcy* genes can be applied to the detection of microcystin-producing cyanobacteria, since generally, nonmicrocystin-producing strains lack *mcy* genes (Börner and Dittmann 2005). Some nonmicrocystin-producing strains of cyanobacteria have been found to possess *mcy* genes or parts of these genes (Neilan *et al*. 1999, Kaebernick *et al.*

2001, Mikalsen *et al.* 2003, Kurmayer *et al*. 2004, Via-Ordorika *et al.* 2004), and thus, the presence of such genomes would not be related to microcystin production. Christiansen *et al*. (2006) found that mutations occur within *mcy* genes of cyanobacteria, indicating that *mcy* genes are as vulnerable as the rest of the genomic DNA. The *mcy* gene sequences of all the known microcystin-producing genera have not yet been resolved, and occurrence of these genera may remain undetected, if genus and *mcy* gene specific primers or probes are used.

The *mcy* gene-specific primers used in polymerase chain reaction (PCR) have been applied *in situ* to identify the presence of microcystin-producing genera of *Microcystis* and *Planktothrix* (Hisbergues *et al*. 2003, Jungblut and Neilan 2006), to detect and study the genotypes of microcystin-producing *Microcystis* (Bittencourt-Oliveira 2003, Dittmann and Börner 2005, Hotto *et al*. 2005, Ouellette *et al*. 2006, Wilson *et al*. 2005, Yoshida *et al*. 2005), and to assess genome numbers of microcystin-producing and nonmicrocystin-producing *Microcystis* (Kurmayer and Kutzenberger 2003, Rinta-Kanto *et al*. 2005) and *Planktothrix* (Kurmayer *et al*. 2004).

MALDI-TOF-MS can be used to detect microcystins from single colonies of cyanobacteria collected *in situ* (Fastner *et al*. 2001, Welker *et al*. 2002). Prior to MALDI-TOF-MS analysis, cyanobacterial colonies may be classified according to their morphological characteristics under a light microscope. It is assumed that cells within a single colony originate from a single cell and represent one strain. Isolation of colonies should be random, since, for example, large colonies have been found to possess *mcy* genes more often than smaller colonies (Kurmayer *et al*. 2003, Via-Ordorika *et al*. 2004). MALDI-TOF-MS has been used to study whether the microcystin production is related to *Microcystis* or *Planktothrix* species classified according to the rules of the ICBN (Fastner *et al*. 2001, Kurmayer *et al*. 2002, 2003, 2004, Via-Ordorika *et al*. 2004, Welker *et al*. 2004) and to identify microcystin variants from single colonies of *Microcystis* (Fastner *et al*. 2001, Kurmayer *et al*. 2002, Via-Ordorika *et al*. 2004, Welker *et al*. 2004, Saker *et al*. 2005a), and *Planktothrix* (Kurmayer *et al*. 2004).

The isolated colonies of microcystin-producing and nonmicrocystin-producing *Microcystis* of lake water were differentiated as well when the rRNA internally transcribed spacer region was amplified and the products analysed with DGGE (Janse *et al*. 2004).

1.2.6. Speculated roles of microcystins

Despite continuous research efforts, the possible role of microcystins has remained unclear. Microcystins are not essential metabolites, since nonmicrocystin-producing strains occur (Sivonen and Jones 1999) and genetically modified microcystin deficient mutants have survived (Dittmann *et al*. 1997, Nishizawa *et al*. 1999). Microcystin-producing cyanobacterial strains contain microcystins in all growth phases. Toxins are thought to be released into the surrounding water, mostly after cell rupture (Sivonen and Jones 1999), and no direct evidence exists that the microcystins are actively exported out of the cells. Microcystins have been shown to be situated particularly around the thylakoids of the *Microcystis* cells (Shi *et al.* 1995, Young *et al.* 2005).

The nonmicrocystin-producing and microcystin-producing cyanobacteria may also synthesize bioactive peptides other than microcystins (Namikoshi and Rinehart 1996, Fastner *et al.* 2001), which may have similar functions as microcystins in the nonmicrocystin-producing cells. In addition, deficiency of certain peptides may be replaced by compensatory production of other corresponding peptides (Repka *et al*. 2004). Fastner *et al.* (2001) analysed a total of 258 isolated *Microcystis* colonies with MALDI-TOF-MS, and all contained some oligopeptides, whereas Welker *et al*. (2004) reported that in 19 colonies out of 165, no peptide metabolites were observed. Many of the oligopeptides produced by cyanobacteria are protease inhibitors (Namikoshi and Rinehart 1996). One of the cyclic depsipeptides, microviridin J (Rohrlack *et al*. 2003), causes trypsin inhibition, which leads to lethal molting disruption in *Daphnia* sp. (Rohrlack *et al.* 2004).

Microcystins have most often been considered deterrants against grazing zooplankton. Negative effects of microcystins and microcystin-producing cyanobacteria have been shown on the survival and feeding of cladocera and copepods (DeMott *et al.* 1991, Rohrlack *et al.* 1999). These effects are evident, since microcystins inhibit serine- and threonine-specific protein phosphatase 1 and 2A activities of all eukaryotic cells. For example, microcystin-LR has also been found to have adverse effects on photosynthesis, seedlings, and sucrose metabolism of terrestrial plants (Falconer *et al*. 1999). The *mcy* genes of cyanobacteria have been found to be ancient and probably predate the metazoan lineage (Rantala *et al.* 2003). Therefore, toxic effects on zooplankton may not be the primary role of microcystins. Microcystins have also been thought to act as chelators in iron scavenging (Utkilen and Gjølme 1995), as an internal storage for N during N deficiency (Kotak *et al*. 2000), in cyanobacterial cell signalling (Dittmann *et al*. 2001), in light adaptation processes (Hesse *et al*. 2001), in inhibition of the carbon-concentration enzyme RuBisCo (Jähnichen *et al*. 2001), and as mediators in colony formation (Kehr *et al.* 2006).

The loss of *mcy* genes seems to be an ongoing process in the long evolution of cyanobacteria (Rantala *et al.* 2004, Jungblut and Neilan 2006). However, many of the common mass occurrence-forming planktonic cyanobacterial strains have maintained the ability to produce microcystins (Sivonen and Jones 1999), while very few benthic and terrestrial cyanobacterial strains are known to produce microcystins (Prinsep *et al*. 1992, Hitzfeld *et al.* 2000, Oksanen *et al.* 2004). This view, however, may be biased, because there is more research on planktonic than on benthic and terrestrial cyanobacteria. It may be speculated that microcystins are advantageous particularly for the pelagic cyanobacteria. This assumption is supported by observations that planktonic *Nodularia* strains isolated from the Baltic Sea produced nodularins, whereas the benthic strains of *Nodularia, Anabaena, Nostoc,* and *Phormidium* were found to produce neither nodularins nor microcystins (Lyra *et al*. 2005, Surakka *et al*. 2005).

1.3. Factors promoting cyanobacterial success

1.3.1. The effects of environmental factors

A number of chemical, physical, and biological factors and their interactions, all of which can be affected by global climate change (Malmaeus *et al*. 2006, Huisman *et al*. 2006), determine the cyanobacterial biomass of a water body (Hyenstrand *et al.* 1998, Dokulil and Teubner 2000, Paerl *et al*. 2001). It is widely accepted that P and N supplies are the most important factors enhancing the growth of cyanobacteria *in situ* (Chorus and Mur 1999). The results of most of the studies in Table 2 support this conclusion, because high cyanobacterial biomasses have generally been associated with high nutrient concentrations *in situ*. The P and N concentrations tested have enhanced growth of *Microcystis* strains (Table 1) and P concentrations have enhanced growth of *Anabaena* (Rapala *et al*. 1993, 1997) in most of the culture experiments. In general, it is thought that nutrient concentrations *in situ* and the concentrations within the cells influence the growth of cyanobacteria more than the N:P ratio of the environment, an explanation which has been put forth to account for the abundance of cyanobacteria (Smith 1983). Several observations do not support the significance of the N:P ratio of cyanobacterial growth (Reynolds 1999, Downing *et al*. 2001, Xie *et al*. 2003b, Rolland *et al*. 2005).

Nutrients may enter the water bodies as diffuse or point-source loading. In addition to internal P loading, which is significant in some lakes, cyanobacteria themselves may indirectly enhance the release of P from sediments (Xie *et al.* 2003a). Human impacts such as run-off from agriculture, waste water discharge, and manipulation of water retention times favour cyanobacteria (Bartram *et al.* 1999, Paerl *et al*. 2001) and alter species composition (Lepistö *et al*. 2004). Domestic waste water promotes the growth of primary producers more than P arriving from field and forest runoff (Ekholm and Krogerus 2003). Other chemical factors such as micronutrients, dissolved inorganic carbon, and dissolved organic carbon concentrations may have effects on cyanobacterial growth (Paerl *et al*. 2001).

Cyanobacterial growth is influenced by physical factors including light, temperature, water turbulence, and mixing. Also water residence time and morphometry of the water body can indirectly influence cyanobacteria. Planktonic cyanobacteria possess gas vesicles and may therefore obtain a vertical position where net photosynthesis is possible (Walsby 1994). In addition, the planktonic cyanobacteria overcome the problem of sinking out of the photic layer due to their gas vesicles. Large cyanobacterial colonies may rise and sink faster than single cells, which are hardly able to alter their vertical position (Walsby 1994). After using enough carbohydrates or synthesizing more gas vesicles to compensate for the buoyancy of the collapsed vacuoles, the cells may ascend again (Walsby 1994).

Grazing, competition, parasitism, and other microbial interactions are biological factors influencing the growth of cyanobacteria. The heterotrophic bacteria, protozoans, and viruses (Fuhrman 1999, Šimek *et al,* 2001, Palenik *et al*. 2003, Lindell *et al*. 2005) have been shown to share complex interactions, and the effects of all of these on the growth of cyanobacteria and the fluxes of organic matter within the microbial loop are being

Table 2. Environmental factors reported to have an association with microcystin (MC) concentrations *in situ*.

Mass occurrence dominated by:	Reported association with microcystin concentration	Reference
Microcystis		
Herbeespoort Dam reservoir, South Africa	Positive with solar radiation and water temperature, negative with PO_4-P.	Wicks and Thiel 1990
Coal Lake in Alberta, Canada	Positive with water temperature, total and dissolved P, and with Secchi depth.	Kotak *et al.* 1993
3 lakes in Alberta, Canada	Positive with total and dissolved P, negative with NO_3-N, no association with water temperature.	Kotak *et al.* 1995
Lake Akersvatnet, Norway	Hepatotoxic *Microcystis* disappeared when NO_3-N concentration decreased.	Utkilen *et al.* 1996
Lake Tuusulanjärvi, Finland	Positive with total N, NO_3 + NO_2-N, total P, negative with Secchi depth.	Lahti *et al.* 1997
Lake Grand-Lieu, France	Positive between a variant of MC-RR and NO_3-N. Negative between MC-LR and a variant of MC-RR with solar radiation.	Vezie *et al.* 1998
Lake Steilacoom, USA	Positive with soluble reactive P.	Jacoby *et al.* 2000
13 lake basins in Alberta, Canada	Positive with total P.	Kotak *et al.* 2000
27-35 water bodies in Germany	Positive with total P, euphotic depth, and ratio of euphotic depth to mixing depth.	Chorus *et al.* 2001
The Kucukcekmece Lagoon, Turkey	Positive with temperature, salinity, total N:P ratio, and light intensity. Negative with water transparency.	Albay *et al.* 2005
The waterways in the San Francisco Bay Estuary, USA	Positive with temperatures and higher Secchi depths. Negative with salinity.	Lehman *et al.* 2005
Lake Tuusulanjärvi, Finland	Positive correlation with cyanobacterial biomass, which showed positive association with total P and N concentration.	Lepistö *et al.* 2005b
64 water bodies in Belgium, Luxembourg, and France	Positive with N:P, Si, and Mg concentrations.	Willame *et al.* 2005
Co-dominance of *Microcystis* and *Anabaena*		
22 lakes in southern Quebec, Canada	Positive with total N and P concentrations.	Giani *et al.* 2005
4 water bodies in the region of Quebec, Canada	Positive with total N. Negative with poor light climate. No association with total P.	Rolland *et al.* 2005
Dominant genera not reported		
Lake Taihu, China	Positive with temperature and trophic status.	Shen *et al.* 2003
241 lakes in Missouri, Iowa, Kansas, and Minnesota, USA	Positive with latitude, total N, and P concentrations. The highest MC concentrations at Secchi depths < 2.5 m.	Graham *et al.* 2004

studied intensively at present (Hoppe *et al*. 2002).

1.3.2. Cyanobacterial P and N uptake

Cyanobacteria actively incorporate orthophosphate-P (P_i) through the transport systems of membranes (Simonis and Urbach 1973). In addition, some phosphorylated sugars and phosphonate are taken up directly (Palenik *et al*. 2003). In cells, P_i may be converted into polyphosphate, a storage compound of P_i (Kornberg 1995), and several granules of polyphosphate may be formed. These storage compounds may be sufficient for several cell divisions (Mur *et al*. 1999). Cyanobacteria can utilise ammonium, nitrite, and nitrate as sole N sources (Tandeau de Marsac and Houmard 1993). Heterocysts are cells that have differentiated from vegetative cells and specialised for N_2-fixation. Heterocystous genera such as *Anabaena* and many nonheterocystous cyanobacteria are capable of N_2-fixation (Tandeau de Marsac and Houmard 1993). Heterocystous genera may differentiate vegetative cells to N-rich resting cells, akinetes, which may survive periods less favourable for growth. The surplus N may be stored as cyanophycin, which may form granules in the cells (Simon 1971). In addition, phycobiliproteins, which are pigment-protein complexes harvesting light, may be used as N sources during nutrient starvation (Schwarz and Grossman 1998). Cyanobacteria have several acclimation responses such as increased synthesis of high affinity transport systems and extracellular enzymes when grown under nutrient deficiency (Schwarz and Grossman 1998).

During the growth season, P_i (Hudson *et al*. 2000) and combined inorganic N concentrations may be low or depleted in pelagic waters simultaneously with variable concentrations of dissolved organic P and N compounds (Münster & Chróst 1990). Cyanobacteria may utilise the nutrients of organic matter through enzymatic depolymerisation (Chróst 1990). The significance of extracellular enzyme activities and dissolved organic P and N compounds on growth of cyanobacteria are not well known. P_i may be hydrolysed by phosphatase activity from such compounds such as polyphosphates, phosphate esters, sugar alcohols, cyclic alcohols, phenols, and amines (Chróst 1991). In most studies, P_i deficiency has increased phosphatase activity (Chróst 1991). In addition, phosphatase activity has been induced by phosphate-containing organic compounds (Whitton *et al.* 1991). Several putative genes encoding alkaline phosphatases exist in the *Synechococcus* strain (Palenik *et al*. 2003).

Dissolved free amino acids may be hydrolysed with aminopeptidase activity. Due to rapid utilisation rates of these acids, concentrations *in situ* have been low (Münster & Chróst 1990). Aminopeptidase activity has been detected in *Synechococcus* strains (Martinez and Azam 1993), and ^{14}C-leucine has been incorporated into cells of *Microcystis* and *Nodularia* (Kamjunke and Jähnichen 2000, Hietanen *et al*. 2002). In the genome of a *Synechococcus* strain, regions encoding functions to exploit a wide range of N substrates have been found (Atanassova *et al*. 2003, Palenik *et al*. 2003).

1.4. Cyanobacterial mass occurrences in freshwater

During cyanobacterial mass occurrence, greenish or reddish colour will be observed in a water body. The cyanobacterial

surface mass occurrences may drift as windblown. Cyanobacteria occur in all freshwaters, but generally their mass occurrences result from proliferation of planktonic cyanobacteria when conditions are favourable. In addition to surface mass occurrences, subsurface mass occurrences have been observed (Lindholm and Meriluoto 1991, Paerl *et al*. 2001).

The frequency of cyanobacterial mass occurrences has increased with accelerated eutrophication (Paerl *et al*. 2001) observed in northwestern Europe and North America from the 1950s until quite recently (Bartram *et al*. 1999). Cyanobacterial mass occurrences typically appear in eutrophic lakes, which either have encountered antrophogenic nutrient loading or are naturally nutrient rich. In addition, some genera such as *Anabaena* may also form mass occurrences in less eutrophic lakes (Lindholm *et al*. 2003, Lepistö *et al*. 2005b), particularly after calm weather conditions. These mass occurrences are generally short-term, whereas in eutrophic lakes, mass occurrences may last for months. In temperate areas, mass occurrences are most prevalent during the late summer and early autumn, whereas in tropical climates mass occurrences may appear during any season (Sivonen and Jones 1999). In addition to the potential health hazards due to the toxins, cyanobacterial mass occurrences may deteriorate the water quality by producing nuisance odour and taste compounds. Furthermore, they may alter the oxygen concentration and the functioning of the food web and lower the aesthetic and recreational value of the water body (Paerl *et al*. 2001).

Typically, mass occurrences are comprised of a single or few cyanobacterial genera such as *Microcystis, Anabaena, Aphanizomenon*, and *Planktothrix* (Sivonen and Jones 1999). The most common genus in Finnish freshwater mass occurrences has been *Anabaena* (Sivonen *et al.* 1990, Lepistö *et al*. 2005b).

1.4.1. Mass occurrences containing microcystins

In surveys conducted all over the world, 1% to 95% of mass occurrences and water samples containing cyanobacteria have been found to be hepatotoxic (Sivonen and Jones 1999, Chorus 2001, Lindholm *et al*. 2003, Kardinaal and Visser 2005, Willame *et al*. 2005). Hepatotoxic mass occurrences have been more common than neurotoxic mass occurrences both in Finland (Sivonen *et al*. 1990) and in other countries (Chorus 2001). It is difficult to understand the effects of environmental factors on the growth of microcystin- and nonmicrocystin-producing cyanobacteria, since the possible role(s) of microcystin are not known. In addition, in only a few studies have the effects of environmental factors on the growth of both microcystin-producing and nonproducing cyanobacteria been studied (Hesse *et al*. 2001, Hesse and Kohl 2001, Böttcher *et al*. 2001). In general, microcystin-containing cyanobacterial mass occurrences are likely to recur as long as the ecological conditions of a water body remain unchanged (Sivonen and Jones 1999, Welker *et al*. 2003, Lepistö *et al*. 2005a), although considerable year-to-year variation has been observed in temperate regions (Lindholm *et al*. 2003).

Numerous studies indicate the importance of nutrient concentrations as regulators of cyanobacterial biomass and community compositions (Rapala and Sivonen 1998, Kotak *et al*. 2000, Chorus *et al*. 2001, Lepistö *et al*. 2005b, Willame *et al*. 2005). A statistical

analysis using the environmental data gathered during mass occurrences in the 1980'es in Finland indicated that the most distinguishing factors among hepatotoxic, neurotoxic, and nontoxic mass occurrences were the dissolved nutrient concentrations (Rapala and Sivonen 1998). Hepatotoxic *Microcystis* mass occurrences were related to higher P_i concentrations than were hepatotoxic and nontoxic *Anabaena* mass occurrences. Positive associations have been reported between microcystin concentrations and the dissolved P and N concentrations, total P and N concentrations, N:P ratio, Si and Mg concentrations, light conditions, water temperatures, salinity, and the trophic status of a water body (Table 2). The simultaneous influence of numerous factors on cellular microcystin concentrations and on the growth of microcystin and nonmicrocystin-producing cyanobacteria complicates the evaluation of the significance of environmental factors *in situ*. Furthermore, it is difficult to distinguish between those environmental factors that promote formation of a mass occurrence and the factors attributable to a mass occurrence.

1.4.2. Microcystin concentrations *in situ*

Due to the drifting and mixing of cyanobacterial cells in water by wind and turbulence, microcystin concentrations may vary, both spatially and in time *in situ* (Ekman-Ekebom *et al.* 1992, Kotak *et al.* 1995, Vezie *et al.* 1998, Welker *et al*. 2003). In addition, environmental factors influence the biomass of microcystin-producing cyanobacteria (i.e., growth yield) as well as cellular microcystin concentrations (Table 1; Sivonen and Jones 1999). Microcystin concentrations *in situ* may vary, due to changes in the abundance of strains with varying cellular microcystin concentrations (Sivonen and Jones 1999). Within a mass occurrence, microcystins have been produced by a single genus or several cyanobacterial genera (Sivonen and Jones 1999, Fastner *et al*. 1999), and nonmicrocystin-producing and microcystin-producing strains within a single genus have co-occurred (Ohtake *et al*. 1989, Vezie *et al*. 1998, Fastner *et al*. 2001, Kurmayer *et al*. 2002, 2003, Bittencourt-Oliveira 2003, Wilson *et al*. 2005).

Although biomass of the potential microcystin-producing cyanobacteria has correlated positively with microcystin concentrations *in situ* (Kotak *et al*. 1995, Kotak *et al*. 2000, Chorus *et al*. 2001, Oh *et al*. 2001, Welker *et al*. 2003), in some lakes the microcystin concentrations have been highest at times other than during the highest cyanobacterial biomass (Watanabe *et al*. 1992, Jähnichen *et al*. 2001, Briand *et al*. 2002, Janse *et al*. 2005, Kardinaal and Visser 2005). In Germany's Lake Müggelsee, the biomass of nonmicrocystin-producing *Microcystis* was thought to be higher during the highest *Microcystis* biomass because the microcystin concentrations per biovolume were lower than during other times (Welker *et al*. 2003). In Lake Wannsee, also in Germany, the proportion of the *mcy* gene containing *Microcystis* genotypes varied from 1% to 38% of the total number of *Microcystis* genotypes from winter to summer (Kurmayer and Kutzenberger 2003). Similarly, in Dutch lakes microcystin and nonmicrocystin-producing *Microcystis* were detected, and nonmicrocystin-producing colonies were thought to be dominant (Janse *et al*. 2004). The microcystin content of dry weight of mass occurrence material has varied from 0 - 19 500 μg g^{-1} (Sivonen and Jones 1999, Kardinaal and Visser 2005).

Total microcystin concentrations as high as 25 000 μg l^{-1} have been detected in the surface waters of lakes (Sivonen and Jones 1999). Microcystin concentrations may be particularly high at the lake shores, owing to the accumulation of cyanobacterial colonies (Welker *et al*. 2003, Kröger and Koehler 2005) and re-immersion (Jones *et al*. 1995) of the extracellular microcystins. Microcystins are relatively persistent compounds (Sivonen and Jones 1999), and extracellular microcystin concentrations as high as 200 μg l^{-1} have been measured in lake water (Sivonen and Jones 1999). Dangerously high extracellular concentrations (up to 1 800 μg l^{-1}) were detected in lake water after algicide treatment chemicals had been spread in order to put an end to cyanobacterial mass occurrence (Jones and Orr 1994). Extracellular microcystins have been found to be more persistent than cellular microcystins *in situ* (Lahti *et al*. 1997). Although the microcystins are persistent compounds, some heterotrophic bacteria are able to degrade these toxins. The biodegradation has been higher in those heterotrophic bacterial assemblages, which had been frequently exposed to microcystin-producing cyanobacteria (Rapala *et al.* 1994, Lahti *et al*. 1998). At least certain strains of *Sphingomonas sp*. (Bourne *et al.* 1996), *Pseudomonas aeruginosa* (Takenaka *et al*. 1997), *Paucibacter toxinivorans* (Rapala *et al*. 2005a), and *Sphingosinicella microcystinivorans* (Maruyama *et al.* 2006) are able to degrade microcystin.

1.4.3. The effects of nutrients on cellular microcystin concentrations

Due to the lack of precise quantification methods to assess the microcystin-producing cyanobacterial cells and the cellular microcystin concentrations *in situ*, it has been difficult to assess the significance of the effects of environmental factors on cellular microcystin concentrations. Therefore, the effects of P, N, and micronutrient concentration as well as light, temperature, salinity, energy charge, and pH on cellular microcystin concentration have been studied by conducting batch and continuous culture experiments with cyanobacterial strains (Table 1; Sivonen and Jones 1999, Bickel and Lyck 2001, Böttcher *et al.* 2001, Hesse and Kohl 2001). Most of these studies are conducted by changing one or a few variables at a time. The N concentrations tested have generally caused an increase in the cellular microcystin concentrations of *Microcystis*, whereas the effects of P have been contradictory (Table 1; Sivonen and Jones 1999). The controversial results of the effects of P on *Microcystis* strains may be explained by the different analysis methods, the concentrations of extracellular microcystins, strain-specific responses, the units of expression, and effects of the growth stage. In general, most studies indicate that the cellular microcystin concentrations of cyanobacteria have been highest under the most favourable growth conditions and that the nutrient concentrations have had less than five-fold effects on cellular microcystin concentration of cyanobacteria (Sivonen and Jones 1999). Thus, environmental factors may influence the microcystin concentration of a water body by influencing both the growth and the abundance of microcystin-producing cyanobacteria and cellular microcystin concentrations (Tables 1, and 2; Sivonen and Jones 1999, Chorus *et al*. 2001).

2. Aims of the study

1) To assess the effects of P and N concentrations and the combined nutrients on the biomass of *Microcystis* strains and an *Anabaena* strain.

2) To assess the effects of P and N concentrations and combined nutrients on the cellular microcystin concentration of *Microcystis* strains cultivated in batch and chemostat cultures.

3) To examine whether continuous culture experiments yield results comparable to batch culture experiments.

4) To design cyanobacterial genus and microcystin synthetase E (*mcyE*) gene-specific primers in order to assess *mcyE* copy numbers of *Microcystis* and *Anabaena in situ* with QRT-PCR (Quantitative Real Time – PCR) and to indicate microcystin-producing genera *in situ* as well as to ascertain the main microcystin producers in two Finnish lakes.

5) To study the effects of P and N concentrations and the combined nutrients on the phosphatase, leucine aminopeptidase, and nitrogen-fixation activities of *Anabaena*.

6) To assess the ecological role of phosphatase and leucine aminopeptidase activities in Lake Hiidenvesi.

3. Materials and methods

Cyanobacterial strains used in this study are presented in Table 3. The procedures and techniques used to study these strains are listed in Table 4 and explained in more detail in the respective papers numbered I, II, III, and IV. The P and N concentrations used in this study (I, II, IV) are summarised in Table 5.

Water samples were collected from the Finnish Lake Tuusulanjärvi from 0 to 2 m depth every second or third week (III) and from four connected natural basins of Lake Hiidenvesi (basins Kirkkojärvi, Mustionselkä, Nummelanselkä, and Kiihkelyksenselkä) in the summer of 1999 (IV). In addition to the surface water samples collected in 1999, water samples were taken from 3 to 5 different depths from the basins of Lake Hiidenvesi on 15 August 2001 (III).

Spearman (I, IV) and Pearson (II) correlations were calculated with SAS Statistical Software for Windows (SAS Institute, Inc, Cary, NC, USA). Central composite design was used to determine the appropriate nutrient concentrations (I, II, IV) and sampling frequency (I, IV) with Matlab Statistical Software for Windows (Math Works, Inc, Natick, MA, USA). Multivariate regression analyses (I, II, IV) were performed to assess the effects of P, N, and the combined nutrients with Matlab Statistical Software for Windows.

Table 3. Purity, microcystin production, and origin of the cyanobacterial strains used in this study. A culture was considered a pure cyanobacterial isolate if no bacterial growth was observed on tryptone-glucose-yeast –agar plates. The strains were provided by the culture collections of PCC (Pasteur Culture Collection, Paris, France), NIES (National Institute for Environmental Studies, Tsukuba, Japan), and NIVA (Norwegian Institute for Water Research, Oslo, Norway). The IZANCYA strains were provided by Professor Vitor Vasconcelos (University of Porto, Portugal). The remaining strains were obtained from the culture collection maintained by the research group of Academy Professor Kaarina Sivonen (University of Helsinki, Finland).

Strain	Purity	Microcystin production	Geographical origin	Paper
Microcystis				
GL260735	+	+	Lake Grand-Lieu, France	I, III
205	+	+	Lake Mallusjärvi, Finland	I, II, III
98	-	+	Lake Pyhäjärvi, Finland	III
GL280646	-	+	Lake Grand-Lieu, France	III
IZANCYA 5	+	+	Lake Mira, Portugal	III
IZANCYA 25	+	+	River Torrão, Portugal	III
NIES102	+	+	Lake Kasumigaura, Japan	III
NIES A 89	+	+	Lake Kawaguchi / Yamanashi, Japan	III
PCC 7941	+	+	Lake Little Rideau, Canada	III
PCC 7806	+	+	Braakman Reservoir, the Netherlands	III
GL060916	+	-	Lake Grand-Lieu, France	I, III
269	+	-	River Raisionjoki, Finland	I, II, III
130	+	-	Lake Säyhteenjärvi, Finland	II, III
Anabaena				
66A	+	+	Lake Kiikkara, Finland	III
90	+	+	Lake Vesijärvi, Finland	III
202A1	+	+	Lake Vesijärvi, Finland	III, IV
202A2/41	+	+	Lake Vesijärvi, Finland	III
NIVA-CYA83/1	+	+	Lake Edlandsvatn, Norway	III
315	+	+	The Baltic Sea (coast), Finland	III
318	+	+	The Baltic Sea (coast), Finland	III
86	-	-	Lake Villinkkalanjärvi, Finland	III
123	+	-	Lake Säyhteenjärvi, Finland	III
14	-	-	Lake Sääksjärvi, Finland	III
PCC 6309	+	-	freshwater, the Netherlands	III
PCC 7108	+	-	intertidal zone, USA	III
PCC 73105	+	-	pond water, Great Britain	III
PCC 9208	+	-	soil, Spain	III
Planktothrix				
49	+	+	Lake Valkjärvi, Finland	III
97	+	+	Lake Maarianallas, Finland	III
213	-	+	Finland	III
NIVA-CYA 126	+	+	Lake Långsjön, Finland	III
NIVA-CYA 127	+	+	Lake Vesijärvi, Finland	III
NIVA-CYA 128/R	+	+	Lake Vesijärvi, Finland	III
45	+	-	Lake Enäjärvi, Finland	III
PCC 6304	+	-	source unknown	III
Nostoc				
152	+	+	Lake Sääksjärvi, Finland	III

Table 4. Methods used in this study.

Parameters	Methods	Paper	References
Dry weight	Dry weight determination	I, II, IV	I, II
Optical density	At 620 and at 750 nm	I, II, IV	I, II
Chlorophyll *a*	Methanol extraction and measurement at 665 nm	I	Tandeau de Marsac and Houmard 1988
Cyanobacterial cell density	Hemocytometer and light microscope	IV	Repka *et al*. 2001
Cyanobacterial biomass	Inverted microscope technique	III, IV	Utermöhl 1958, Tallberg *et al.* 1999
Protein concentration	Lowry method using bovine serum albumin as a standard	I	Herbert *et al.* 1971
Microcystin concentration	HPLC	I	Rapala *et al*. 1994
Microcystin concentration	HPLC	II, III	Repka *et al*. 2001, III
Microcystin concentration	ELISA	III	Instructions of Strategic Diagnostics, Inc.
Purity testing	Tryptone-glucose-yeast extract – agar plates	I, II	Atlas 1993
Purity testing	DAPI staining and epifluorescence microscope	II	Kepner and Pratt 1994
P concentration	Molybdate-ascorbic acid methods	II	SFS 3025 1986, SFS 3026 1986
N concentration	Oxidation with peroxodisulfate	IV	SFS 3031 1990
Cellular C and N concentrations	LECO CHN-900 analyzer	II, IV	Liao 1993, Instructions of the LECO corporation
N-fixation rate	Acetylene reduction	IV	Burris 1972, IV
Aminopeptidase activity	MUF-phosphate as substrate and fluorometer	IV	Hoppe 1993, Wittmann *et al*. 2000
Phosphatase activity	Leucine-AMC as substrate and fluorometer	IV	Hoppe 1993, Wittmann *et al*. 2000
DNA concentration	260 nm	III	III
DNA isolation	Hot phenol-chloroform-isoamyl alcohol	III	Giovannoni *et al*. 1990
DNA purification	Prep-A-gene DNA purification kit	III	Instructions of Bio-Rad
Quantification of gene copy numbers	QRT-PCR	III	Heid *et al.* 1996, Instructions of Roche Diagnostics
Specificity testing	PCR and agarose gel electrophoresis	III	III

Table 5. The P and N concentrations used in culture experiments in this study.

P (mg l^{-1})	N (mg l^{-1})	Paper
0.05 – 5.5	0.84 – 84	I
0.025 – 1.2	2.0 – 84	II
0.055 – 5.5	1.05 – 84	IV

4. Results and discussion

4.1. Cyanobacterial biomass indicators

Dry weight and optical density had statistically significant positive correlations (r = 0.91 – 0.96) in the axenic batch and chemostat cultures of this study (I, II, IV). In addition, in batch cultures of *Anabaena* 202A1 dry weight correlated statistically significantly with cell numbers (r = 0.94) (IV). Hence, the dry weight was used as a unit of biomass, i.e., growth yield, in all culture experiments (I, II, IV).

In the axenic chemostat cultures in this study, the cellular C concentration of *Microcystis* strains was constant under varying nutrient concentrations (Fig. 7C in II), whereas in batch and some continuous cultures, an increase of C storage compounds was observed when cyanobacteria were grown under P or N deficiency (Grossman *et al*. 1994). In addition, the growth rate has been found to influence the cellular weight and volume of *Microcystis* cells when grown under N deficiency (Long *et al*. 2001). Since storage compounds may have effects on the cellular weight of cyanobacteria, cell numbers may be better indicators of cyanobacterial biomass than dry weight. Cell counting with techniques such as flow cytometry could be useful (Dignum *et al*. 2005). When applying sedimentation cuvettes in cell number counting, methodical limitations such as irregular sedimentation of the cells in the counting chambers due to the buoyancy of the cells, the length of cyanobacterial filaments, and difficulties in determining the adjacent cells when counting cells of a genus such as *Planktothrix* may be encountered. Böttcher *et al*. (2001) reported differences up to 1.5-fold among five replicate cell counts of *Microcystis aeruginosa*. Cell counters combined with analyser systems are accurate and may be used to measure the biomass of cyanobacteria cultivated in cultures (Wiedner *et al*. 2003).

Dry weight is not a suitable cyanobacterial biomass indicator *in situ* due to the presence of other plankton and particles in lake water. Cyanobacterial biomass *in situ* may be assessed by counting cyanobacterial cells (Figs. 4, 5, and Table 3 in III) or filament lengths and by converting the data into biovolume, and thereafter expressing the biomass as cyanobacterial wet weight (Fig. 1, Tables 2, and 3 in IV) (Utermöhl 1958). However, in addition to methodical limitations in cell number counting, the transformation of cell numbers and shapes into biovolumes may introduce error. Flow cytometry has been used successfully to determine cyanobacterial biomass *in situ* as well as in cultures (Dignum *et al*. 2005).

Chlorophyll *a* and phycobiliprotein concentrations have been used to assess cyanobacterial biomass *in situ* (Parésys *et al*. 2005, Niemi *et al*. 2004), although it is known that concentrations of both of these may be influenced by environmental factors (Schwarz and Grossman 1998). Environmental factors such as N deficiency have been shown to influence cellular protein concentrations (Grossman *et al*. 1994) similar to the findings in this study (Table 2 in I). The N concentrations tested increased cellular protein concentration of *Microcystis* strains, while cultivation time decreased the concentration (Table 2 in I). The P supply decreased the cellular protein concentration of microcystin-producing strains and increased that of nonmicrocystin-producing *Microcystis* (Table 2 in I). In addition, higher growth rates increased cellular

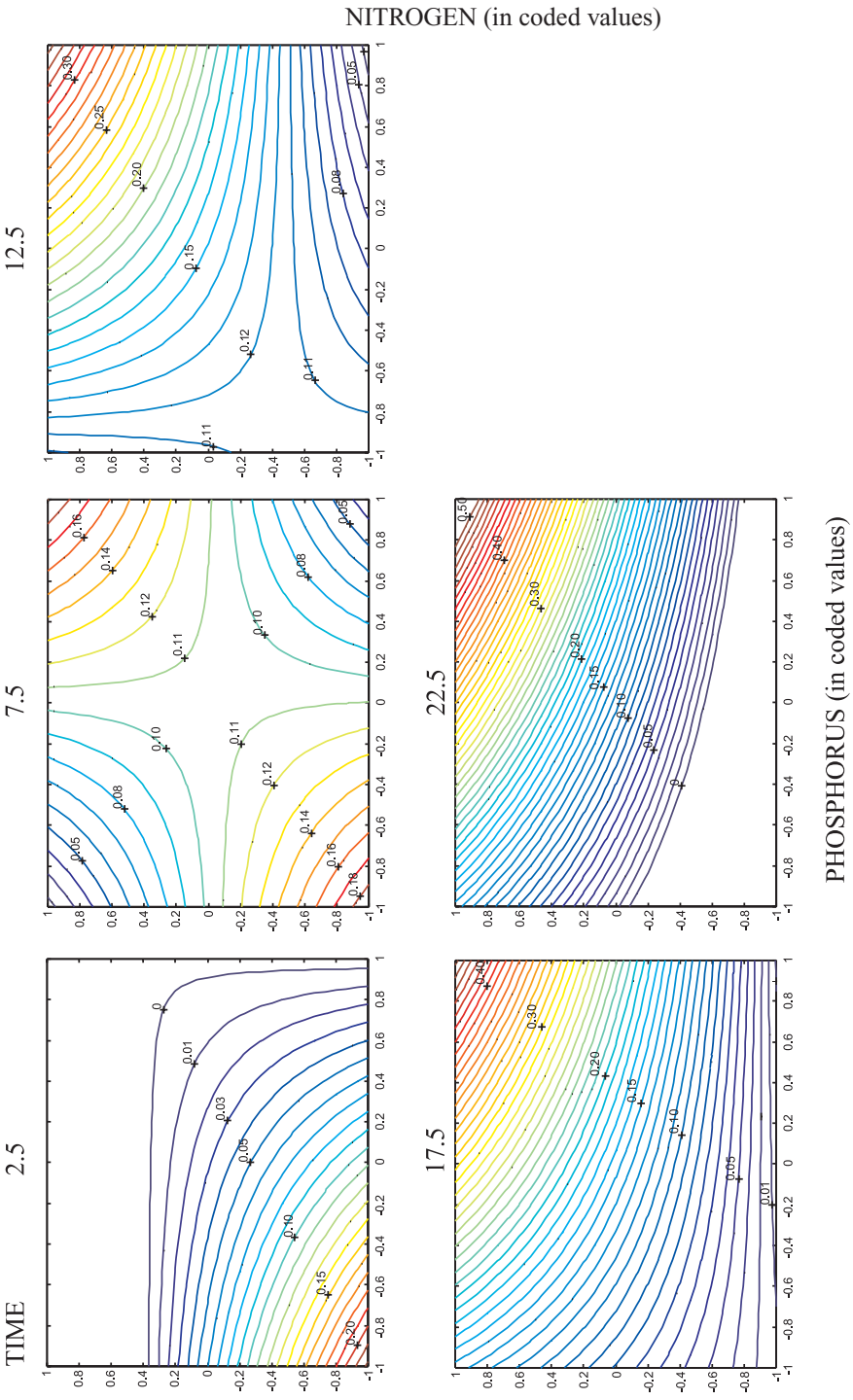

Fig. 2. Contour plots describing biomass (mg l^{-1} dry weight) of microcystin-producing *Microcystis* 205 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

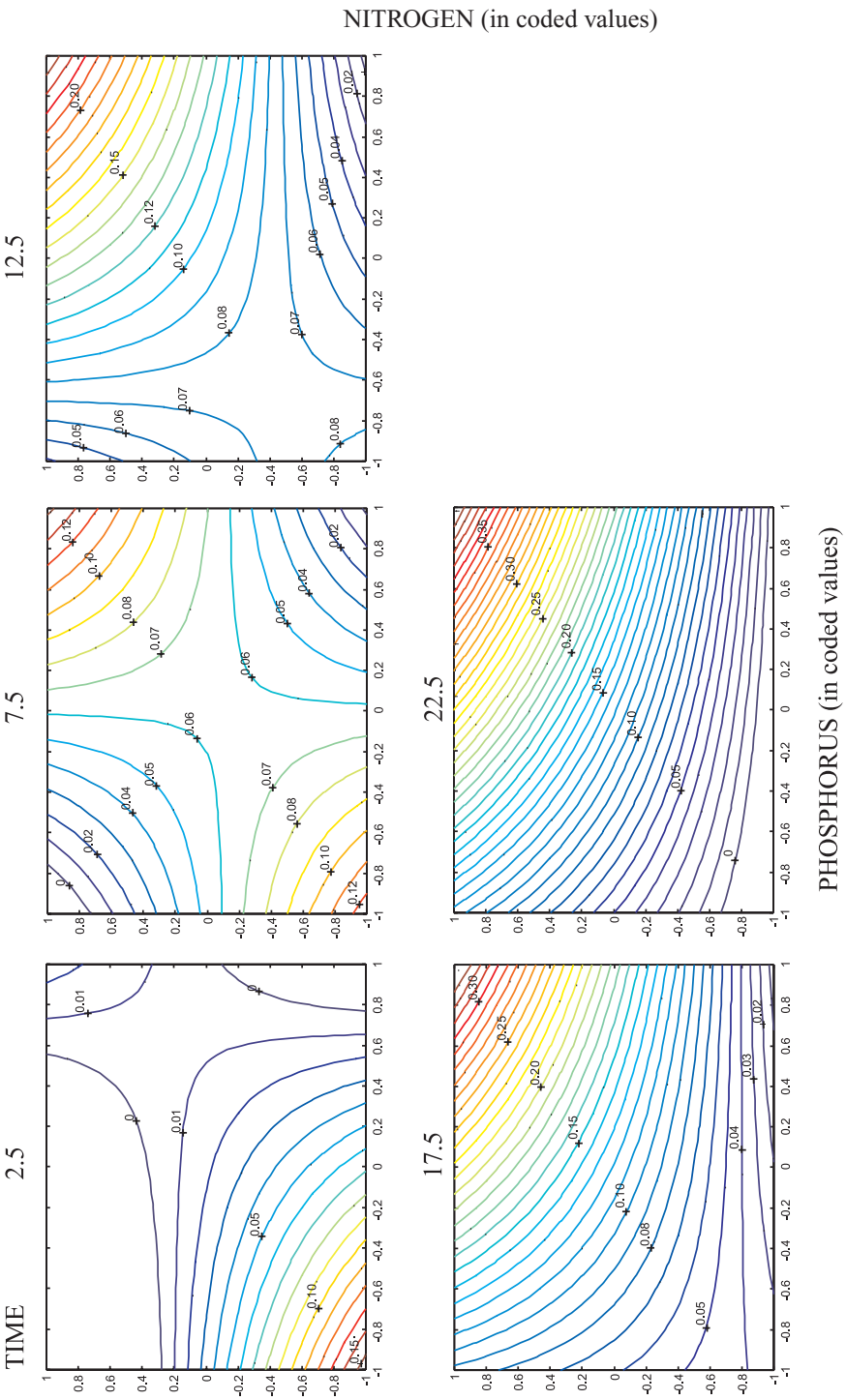

Fig. 3. Contour plots describing biomass (mg l^{-1} dry weight) of microcystin-producing *Microcystis* GL260735 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

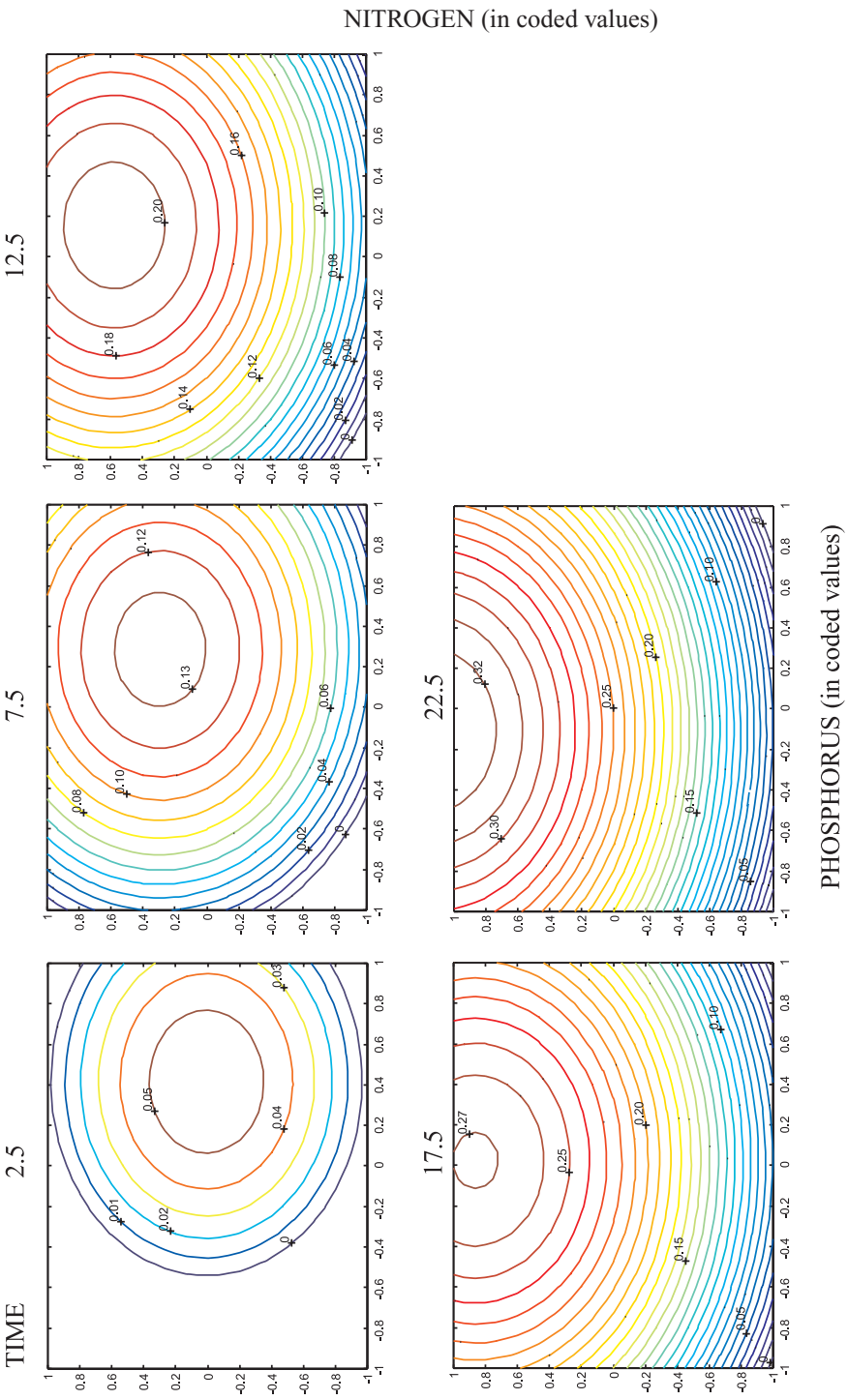

Fig. 4. Contour plots describing biomass (mg l^{-1} dry weight) of nonmicrocystin-producing *Microcystis* 269 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

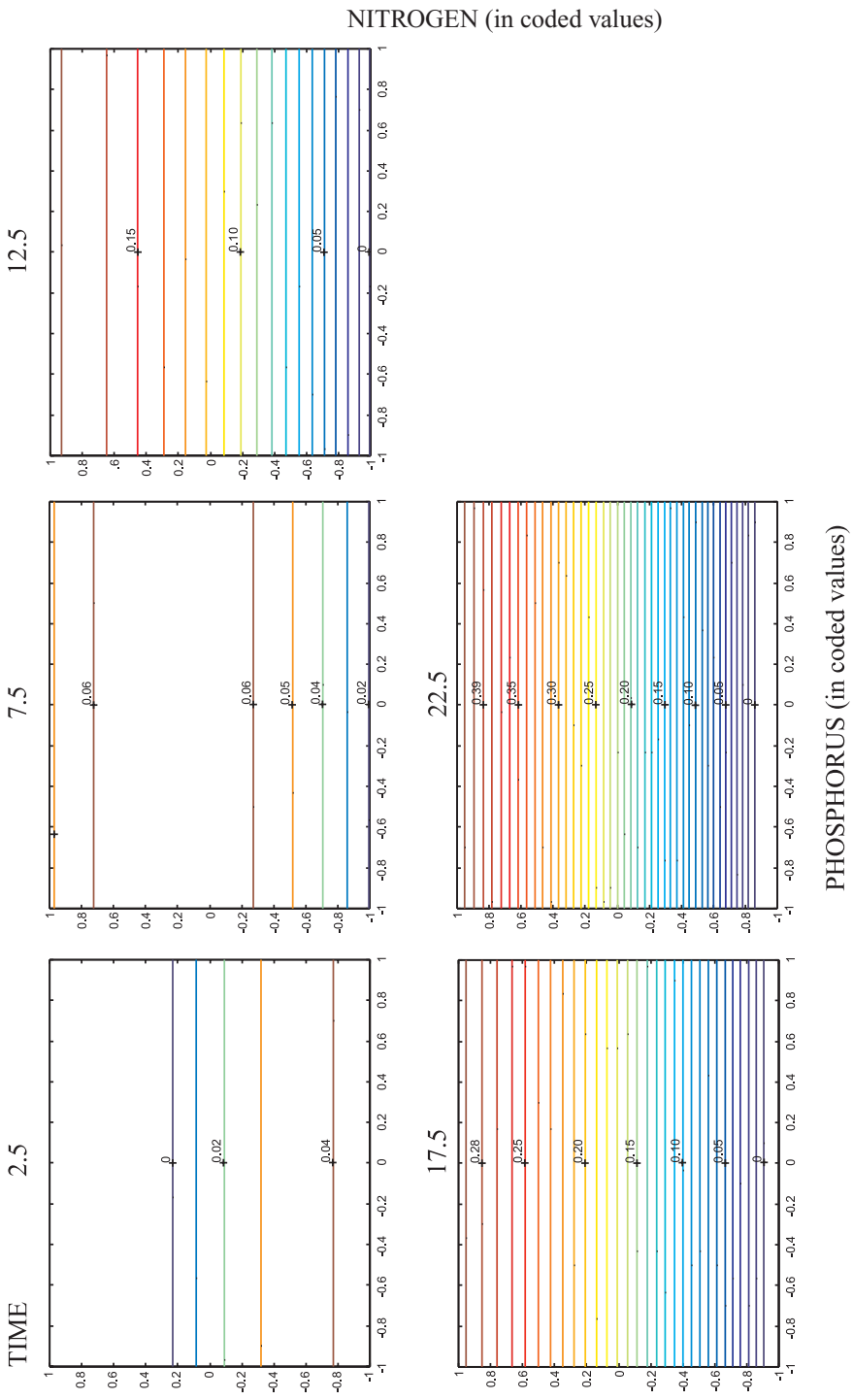

Fig. 5. Contour plots describing biomass (mg l^{-1} dry weight) of nonmicrocystin-producing *Microcystis* GL060916 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

protein concentrations when grown in P deficiency (Oh *et al.* 2000). Cellular protein concentrations cannot be regarded as constant biomass indicators.

4.2. Effects of P and N

4.2.1. Biomass of *Microcystis* and *Anabaena*

The tested P and N concentrations increased the biomass of all *Microcystis* strains studied in batch (Table 2 in I; Figs. 2-5) and chemostat cultures (Table 1 in II). All results of N were statistically significant, and high N concentrations seemed to be necessary for growth of all *Microcystis* strains (Fig. 1 in I; Fig. 2 in II). The P concentrations increased statistically significantly the growth yield of all *Microcystis* strains in chemostats (Table 1 in II) and the growth yield of microcystin-producing strains in batch cultures (Table 2 in I). Interaction of P and N increased statistically significantly the growth yield of microcystin-producing strains (Table 2 in I, Table 1 in II, mathematical models in Figs. 3A, and B in I; Fig. 3A in II) and that of the nonmicrocystin-producing 269 strain grown in chemostat cultures (Table 1 in II, and the mathematical model in Fig. 3B in II). The highest nutrient concentrations (1.2 mg l^{-1} of P and 84 mg l^{-1} of N; Table 5) used in chemostat cultures to cultivate *Microcystis* strains were much higher than the total P and N concentrations generally observed *in situ* (Wetzel 1983). However, even higher biomass could have resulted if higher nutrient concentrations had been used in chemostat cultures (mathematical models in Figs. 3A, B, and C in II).

As in this study, all P and N concentrations tested elsewhere have increased the biomass of most other *Microcystis* strains cultivated in batch and continuous cultures (Table 1; Sivonen and Jones 1999). However, in batch cultures the highest biomass of the nonmicrocystin-producing *Microcystis* 269 was observed within studied nutrient concentrations (0.05 – 5.5 mg l^{-1} of P and 0.84 – 84 mg l^{-1} of N) (Fig. 2C in I), indicating that some factor other than these nutrients limited growth. The P concentration did not increase the growth yield of nonmicrocystin-producing *Microcystis* GL060916 in batch cultures according to the mathematical model (Fig. 2D in I), possibly indicating that this strain could grow with low P concentrations or that some other factor (e.g., light or carbon dioxide) limited growth. Similarly, in the batch cultures of Song *et al*. (1998) and Lee *et al*. (2000), microcystin-producing *Microcystis* biomass was not highest with the highest P concentrations. Experiments with *Microcystis* in continuous cultures give more reliable information than batch culture studies.

The P concentration increased the growth yield of *Anabaena* 202 A1, whereas N concentration did not (Table 3 in IV). The high N concentrations may even have reduced growth (Fig. 4 in IV), although the effect of N was not statistically significant (Table 3 in IV). These results are in accordance with the findings of the N_2-fixation capable cyanobacterial genera *Anabaena*, *Aphanizomenon*, and *Nodularia* (Rapala *et al*. 1993, 1997, Lehtimäki *et al*. 1997, Repka *et al*. 2001). The N_2-fixation requires the activities of numerous enzymes, and in general, it is thought that inorganic-combined N compounds are preferable if available (Flores and Herrero 1994). The *Anabaena* strain cultivated in this study (IV) has been maintained in combined inorganic N-free media since its isolation and may be adapted to fix N_2. On the other hand, N_2-fixation ceased in the batch cultures during the exponential

growth phase, indicating ability of this strain to respond to environmental conditions (Table 3; Fig. 6 in IV). Thus, in accordance with Rapala *et al*. (1997), the N concentration influences the growth of N_2-fixing and non-N_2-fixing cyanobacteria differently.

4.2.2. Biomass of microcystin and nonmicrocystin-producing *Microcystis* strains

At high nutrient concentrations, the biomasses of microcystin-producing *Microcystis* were higher than those of nonmicrocystin-producing strains in batch (Figs. 2-5; Fig. 2 in I) and chemostat cultures (Figs. 1, and 3 in II). In chemostat cultures, the biomass of microcystin-producing *Microcystis* 205 was 32% higher than that of two nonmicrocystin-producing strains in the medium with the highest nutrient concentrations, 1.2 mg l^{-1} of P and 24 mg l^{-1} of N (Fig. 1 in II). Similarly, the biomass of the nodularin-producing *Nodularia* strains was higher than that of the nonnodularin-producing strain with the highest studied P concentrations (0.6 and 1.0 mg l^{-1}) (Lehtimäki *et al*. 1994). It seems that at high P concentrations microcystin- and nodularin-producing strains had higher growth yields than the nontoxic strains. However, differences between the growth of microcystin-producing and nonproducing strains in varying nutrient concentrations were not statistically studied (I, II).

At low nutrient concentrations biomasses of all *Microcystis* strains were comparable in chemostat cultures (Figs. 1, and 3 in II), whereas the biomass of nonmicrocystin- producing *Microcystis* was higher compared to microcystin-producing strains in batch cultures (Figs. 2-5; Fig 2 in I). In batch cultures, lower N concentrations were studied than in chemostat cultures, and it was suggested that nonmicrocystin- producing strains have lower nutrient demands than microcystin-producing strains. The microcystin biosynthesis is considered to be energy- and nutrient- consuming (among others C, N, and P) compared to ribosomal peptide synthesis. Perhaps nonmicrocystin-producing strains grew better than microcystin-producing strains at low nutrient concentrations (Figs. 2-5; Fig 2 in I), since nonmicrocystin-producing strains utilised energy and nutrients for their growth instead of using microcystin biosynthesis. The *Microcystis* strains cultivated in this study may have differed in other features besides microcystin production, and it is possible that observed responses in growth yields may have resulted from other characteristics of these strains. If we assume, however, that the differences in biomasses were due to the presence of microcystins, we may hypothesise the roles of microcystins.

In the chemostat cultures in this study, *Microcystis* strains may have grown under light deficiency in addition to P deficiency due to self-shading of the cells (II). Light may offer an explanation of why microcystin-producing strains had higher biomass than nonmicrocystin-producing strains at high nutrient concentrations (Fig 2 in I; Fig. 1 and 3 in II), if microcystin-producing strains grow better at lower-light intensities than nonmicrocystin-producing strains. Böttcher *et al*. (2001) found that an increase in light intensities resulted in higher growth rates of microcystin-producing *Microcystis* and *Planktothrix* strains than of nonmicrocystin-producing strains. A microcystin-deficient *Microcystis* mutant was found to contain less chlorophyll *a* and other pigments and also showed differences in cellular

structures under light-limiting conditions compared to the wild type (Hesse *et al*. 2001). However, growth of the mutant and wild type did not differ under studied light intensities, 4 - 110 µmol photons m^{-2} s^{-1}, in nutrient-saturated media. Cyanobacterial diversity is attributed to the significance of characteristics for their growth (Rocap *et al*. 2003, Stomp *et al*. 2004). It remains to be discovered whether microcystin production is among these characteristics.

4.2.3. Cellular P and N of microcystin and nonmicrocystin-producing *Microcystis* strains

Increasing P concentrations statistically significantly increased cellular P concentration of all studied *Microcystis* strains cultivated in chemostats (Table 1 in II). Similar to a study of Hesse and Kohl (2001), cellular P concentrations were higher in non-P limiting treatments than in P-limiting treatments. Increasing N concentrations decreased the cellular P of all *Microcystis* strains at low P concentrations, although the response of N to cellular P concentration was less important than the responses of P (Table 1 in II). The cellular P concentration was highest with high P and low N in microcystin-producing *Microcystis* 205 and with high P and high N in nonmicrocystin-producing *Microcystis* 130 and 269 (the coefficients of the interactive effect of P and N in Table 1 in II). These results were statistically significant only for nonmicrocystin- producing strains. Thus, the microcystin-producing strain was able to produce more biomass with lower cellular P concentrations than nonmicrocystin-producing strains under P deficiency at high ambient nutrient concentrations (Table 1 in II). In the study by Hesse and Kohl (2001), the cellular P concentrations of the *Microcystis* strains grown under P limitation did not differ between the microcystin and nonmicrocystin- producing strains. Clearly, the significance of cellular nutrient concentrations on the growth of microcystin and nonmicrocystin-producing cyanobacteria requires more research.

In general, increasing P and N concentrations in the medium increased the cellular N concentration of the *Microcystis* in the chemostat cultures in which growth was P deficient (Table 1 in II). Similarly, Hesse and Kohl (2001) observed that the cellular N concentrations of the six *Microcystis* strains were higher in the non-N limiting treatments than in the N limiting. The cellular N:P ratio was found to correlate positively with cellular microcystin concentrations under P-limited growth by Oh *et al*. (2000). The lowest cellular N concentration was found in the nonmicrocystin-producing strains with low P and low N concentrations, whereas in the microcystin-producing strains the N concentration did not have any effect on the cellular N concentration at low P concentrations. The cellular N content increased as a response to the same factors as the cellular microcystin concentration (P, N, and their interactive effect in Table 1 in II). These results are supported by the findings of Downing *et al*. (2005), who concluded that the microcystins were produced as a function of the cellular N status. In their study, the cellular microcystin concentrations correlated with the cellular protein concentrations in batch cultures under nonlimiting conditions (Downing *et al.* 2005). In our study (II), the average cellular N concentration of the two nonmicrocystin-producing *Microcystis* strains was higher than that of the microcystin-producing *Microcystis*, and thus, the responses were

more likely strain-specific than related to the microcystin production. The cellular N concentrations of the *Microcystis* strains under N-limiting growth did not differ between the microcystin and nonmicrocystin-producing strains in the study by Hesse and Kohl (2001).

4.2.4. Total microcystin concentration

Increasing P and N concentrations and interaction of the nutrients increased total microcystin concentration (μg l^{-1}) of the *Microcystis* strains grown in batch (Table 2 in I) and continuous cultures (Table 1 in II), a consequence of the increase of the biomass. Microcystins were detected in all of the nutrient concentrations studied, although the mathematical model describing the total (Fig. 5 in II) and cellular microcystin concentrations (Fig. 6 in II) suggested low microcystin concentrations at high N and low P concentrations and *vice versa*. Similar to this study, microcystin-producing strains have produced microcystins in all environmental conditions and all growth phases studied to date (Tables 1 and 6; Sivonen and Jones 1999).

4.2.5. Cellular microcystin concentration

In chemostat cultures, increasing P and N concentrations and interaction of the nutrients increased cellular microcystin concentration of *Microcystis* 205 (Tables 1, and 6; Figs. 6, and 7; Fig. 4 in II). The combined nutrients also increased cellular microcystin concentrations of the strains *Microcystis* 205 and GL260735 cultivated in batch cultures (Table 2, and Fig. 3 in I). These results are in accordance with the results of most other studies the majority of which have shown that these environmental factors influence cellular microcystin concentration (Table 1; Sivonen and Jones 1999). In addition, results of this study (Table 2 in I; Table 1 in II) support the general view that cellular microcystin concentrations are highest under the most favourable growth conditions (Sivonen and Jones 1999). In general, the environmental factors tested have shown less than five-fold effects on cellular microcystin concentration (Tables 1 and 6; Sivonen and Jones 1999). In addition, cellular microcystin concentrations may vary among different strains grown in identical conditions just as much as the effects of environmental factors may influence the cellular microcystin concentration of a strain. The effects of environmental factors on the biomass of microcystin-producing strains are probably more important than the effects on cellular microcystin concentration with regard to the total microcystin concentration of a water body.

Cellular microcystin concentration and biomass of *Microcystis* 205 had a statistically significant positive correlation when grown in chemostat cultures (II). In batch cultures, high cellular microcystin concentrations were observed at high nutrient concentrations (with high biomass) similar to chemostats and also at low nutrient concentrations (with low biomass) (Figs. 2A and B, Figs. 3A and B in I). One possible explanation for why the cellular microcystin concentration was high at low nutrient concentrations and low biomass in batch cultures may be that the highest cellular microcystin concentration has usually been detected during the logarithmic or early stationary growth phase. It is possible that different growth stages in different media at the time of comparison obscured interpretation of the results. In chemostat cultures, the growth

Table 6. The effects of P and N concentrations on cellular microcystin concentration (MC) measured with HPLC of the *Microcystis* strains grown in cultures.

Studied nutrient Concentrations	Strain	Effects on cellular microcystin concentration (µg mg^{-1} dry weight)		Other growth conditions	Paper
Phosphorus		**With high N**	**With low N**		
0.05-5.5 mg P l^{-1}	GL260735	0.8-fold MC at 5.5 mg l^{-1} than at 0.05 mg l^{-1}	0.2-fold lower MC at 5.5 mg l^{-1} than at 0.05 mg l^{-1}	Batch cultures, 25 d Temperature, 20 ºC Light, 25 µmol m^{-2} s^{-1}	I
0.05-5.5 mg P l^{-1}	205	1.7-fold higher MC at 5.5 mg l^{-1} than at 0.05 mg l^{-1}	0.4-fold lower MC at 5.5 mg l^{-1} than at 0.05 mg l^{-1}	Batch cultures, 25 d Temperature, 20 ºC Light, 25 µmol m^{-2} s^{-1}	I
0.025-1.2 mg P l^{-1}	205	5-fold higher MC at 1.2 mg l^{-1} than at 0.025 mg l^{-1}	Higher MC at 0.025 mg l^{-1}	Chemostat, Growth rate, 0.08 d^{-1} Temperature, 20 ºC Light, 15 µmol m^{-2} s^{-1} 18 h	II
Nitrogen		**With high P**	**With low P**		
0.84-84 mg N l^{-1}	GL260735	4.7-fold higher MC at 84 mg l^{-1} than at 0.84 mg l^{-1}	1.2-fold higher MC at 84 mg l^{-1} than at 0.84 mg l^{-1}	Batch cultures, 25 d Temperature, 20 ºC Light, 25 µmol m^{-2} s^{-1}	I
0.84-84 mg N l^{-1}	205	2.2-fold higher MC at 84 mg l^{-1} than at 0.84 mg l^{-1}	0.5-fold lower MC at 84 mg l^{-1} than at 0.84 mg l^{-1}	Batch cultures, 25d Temperature, 20 ºC Light, 25 µmol m^{-2} s^{-1}	I
2-84 mg N l^{-1}	205	Higher MC at 84 mg l^{-1}	0.5-fold lower MC at 84 mg l^{-1} than at 0.84 mg l^{-1}	Chemostat, Growth rate, 0.08 d^{-1} Temperature, 20 ºC Light, 15 µmol m^{-2} s^{-1} 18 h	II

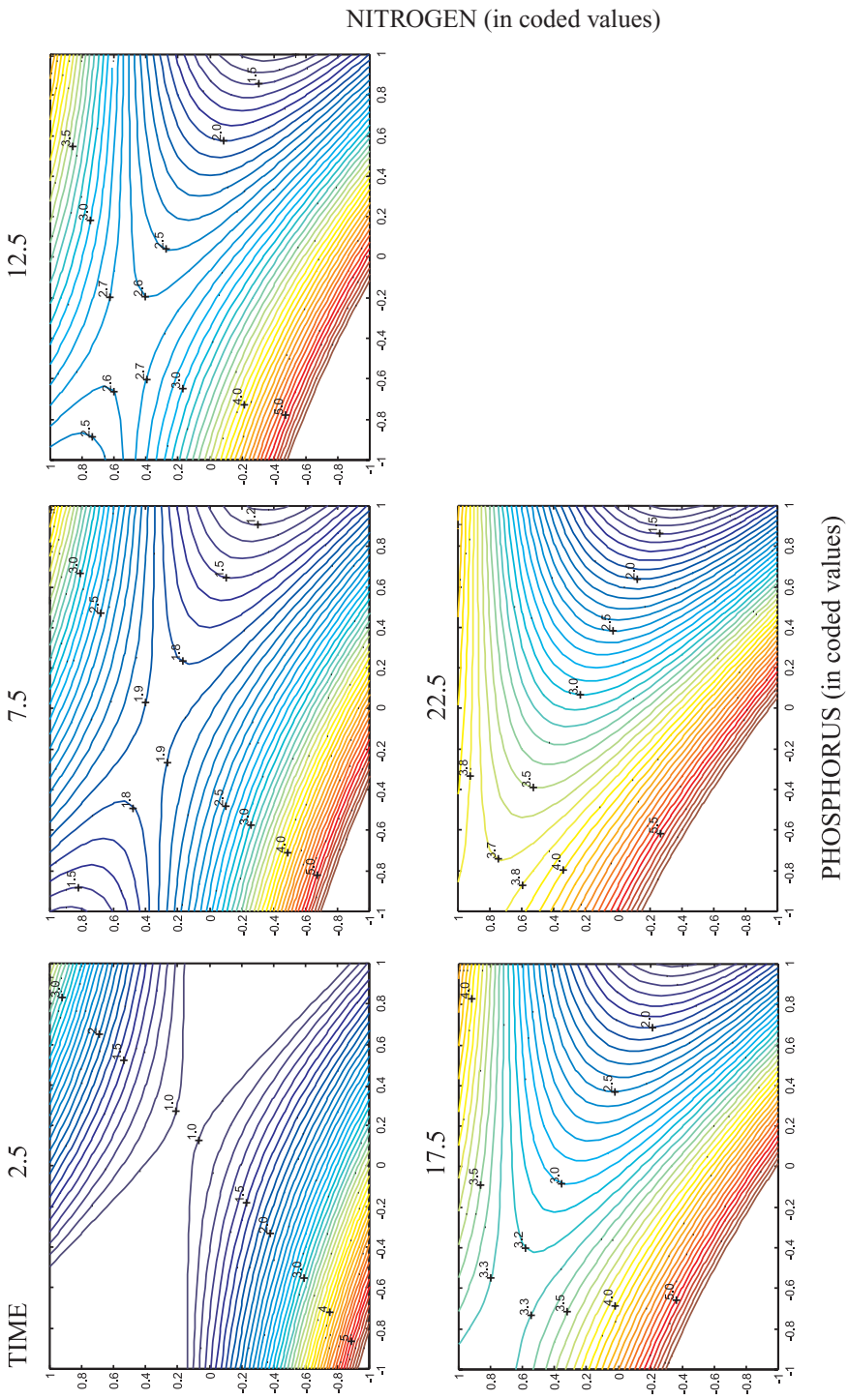

Fig. 6. Contour plots describing cellular microcystin concentration (µg ml^{-1} dry weight) of *Microcystis* 205 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

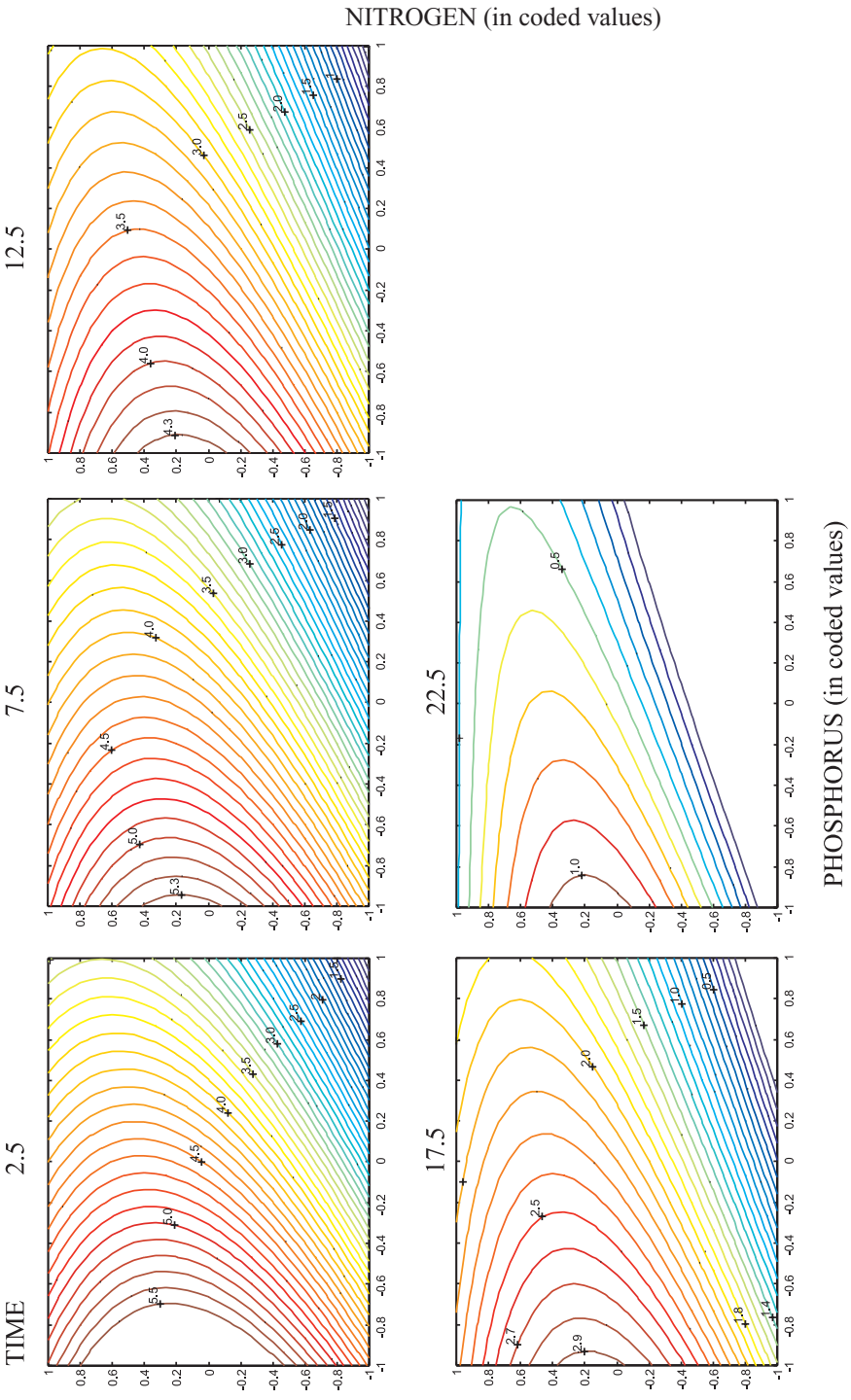

Fig. 7. Contour plots describing cellular microcystin concentration (µg ml^{-1} dry weight) of *Microcystis* GL260735 at different P (0.05 – 5.5 mg l^{-1}), N (0.84 – 84 mg l^{-1}), and times (2.5 – 22.5 d) during batch culture experiments. Nutrient concentrations and times were linearly transformed, i.e., coded, using equation 1. presented in paper I.

phase of the population can be maintained identically in all media.

4.2.6. Extracellular microcystin concentration

Extracellular microcystins were observed in all of the chemostat cultures of *Microcystis* 205, although most of the microcystins were within the cells, similar to the findings of other continuous culture studies conducted with *Microcystis* strains (Böttcher *et al*. 2001, Long *et al*. 2001, Wiedner *et al.* 2003). Cultivation time, P, and N concentrations increased statistically significantly the extracellular microcystin concentrations in batch cultures (Table 2 in I), probably because the extracellular microcystin concentration mostly depends on the total microcystin concentration. In general, it is thought that microcystins are released into the surrounding water after cell rupture (Sivonen and Jones 1999). In the chemostat study, the highest proportion of extracellular microcystins of total microcystins was found at the lowest nutrient concentrations (Fig. 5 in II). Pearson *et al*. (2004) have suggested that the gene *mcyH* functions as a microcystin exporter based on a phylogenetic analysis. However, no direct evidence exists that this function exports microcystins out of the cells.

4.3. Assessment of microcystin producers

4.3.1. Genus-specific microcystin synthetase E gene primers

A genetic method to identify microcystin-producing cyanobacterial genera was developed, since it is not possible to separate the microcystin-producing and nonmicrocystin-producing cyanobacteria with a light microscope. The *mcyE* gene reverse primers designed in this study (Table 1 in III) and the forward primer presented by Rantala *et al.* (2004) were cyanobacterial genus-specific, since a single amplification product was observed when genomic DNAs of microcystin-producing *Microcystis* or *Anabaena* strains were used for templates in PCR (Table 2 in III). In QRT-PCR three *Microcystis* and three *Anabaena* strains showed genus-specific melting temperatures (Table 5 in III). The microcystin production of *mcyE*-containing strains was verified with HPLC (Table 2 in III). The *mcyE* gene encodes the glutamate-activating adenylation domain, and all the microcystin variants possess the D-glutamate (Sivonen and Jones 1999). The α-carboxyl group of D-glutamate has been shown to be essential for toxicity (Namikoshi *et al.* 1992). Jungblut and Neilan (2006) presented *Microcystis, Anabaena, Planktothrix, Nostoc,* and *Phormidium* genus-specific primers to amplify the aminotransferase domain of *mcyE.* Presence of *mcyE* copies of *Microcystis* and *Planktothrix* were indicated *in situ* (Jungblut and Neilan 2006).

4.3.2. QRT-PCR assay

In this study, the *Microcystis* and *Anabaena* genera and the *mcyE*-gene specific primers were amplified with QRT-PCR to indicate potential microcystin-producing genera and to assess the abundance of the producers. The *mcy* genes were used as surrogates for microcystin-producing cyanobacterial genomes, since generally nonmicrocystin-producing strains lack *mcy* genes (Börner and Dittmann 2005). It is believed that only a single copy of the *mcy* gene cluster is present within a single cyanobacterial genome, because the microcystin-deficient mutants were successfully constructed

via transformation (Dittmann *et al*. 1997, Nishizawa *et al*. 1999). External standards for quantification were prepared using the DNA of three *Microcystis* and three *Anabaena* strains (Fig. 1 in III). Since the genome sizes of strains used to construct standards were unknown, the sizes were estimated based on reported genome sizes of other strains of a corresponding genus. The determination of *mcyE* copy numbers *in situ* was calculated by using external standards that gave the highest and the lowest copy numbers (Figs. 2, and 3 in III).

4.3.3. Dominant microcystin producers in two Finnish lakes

It is likely that *Microcystis* was the main microcystin-producing cyanobacterial genus at Lake Tuusulanjärvi in the summer of 1999, since *Microcystis mcy*E copy numbers were 12 to 91 times more abundant than those of *Anabaena* (Fig. 2 in III). This result is in accordance with other findings that have shown positive correlations between hepatotoxic activities and microcystin concentrations with the biomass of *Microcystis* in Lake Tuusulanjärvi (Ekman-Ekebom *et al*. 1992, Lahti *et al*. 1997, Lepistö *et al*. 2005a). *Microcystis mcy*E copy numbers were also more abundant than those of *Anabaena* in the basin Kiihkelyksenselkä of Lake Hiidenvesi (Fig. 3 in III). Most *Anabaena* cells were probably nonmicrocystin-producing in Kiihkelyksenselkä, since the *Anabaena* cell numbers were higher than those of *Microcystis* (Fig. 5 in III), and yet *Microcystis mcy*E copy numbers were more abundant than those of *Anabaena.* The *Microcystis* and *Anabaena mcyE* copy numbers were rather similar in the Nummelanselkä and Mustionselkä basins. The *mcy*E copy numbers of both *Microcystis* and *Anabaena* were below or near the detection limit in Lake Hiidenvesi's most eutrophic basin, Kirkkojärvi (Fig. 3 in III).

4.3.4. The *mcyE* copy numbers, cell numbers, and microcystin concentrations

The *mcyE* copy numbers obtained were higher than the cell numbers calculated with a microscope, as similarly observed in Lake Wannsee (Kurmayer and Kutzenberger 2003) and Lake Erie (Rinta-Kanto *et al*. 2005). One explanation may be that there were several genomes within the cells. At least prior to cell division, the genome is doubled. In addition, the estimated genome sizes of the strains used to construct external standards were based on reported genome sizes. An important prerequisite for quantification with QRT-PCR is that amplification efficiencies between external standards and samples are similar. The presence of PCR inhibitors in samples and competition for primer annealing sites between primers and homologous sequences may be traced with QRT-PCR by diluting the sample and comparing the detected copy numbers with the predicted copy numbers of diluted samples (Table 4 in III). As shown in this study (Figs. 1, 2, and 3 in III), varying amplification efficiencies of standards substantially influence the number of assessed *mcyE* copies.

Environmental conditions, growth rate, and growth phase all influence cellular microcystin concentrations (Table 1; Sivonen and Jones 1999, Böttcher *et al*. 2001). In addition, cellular microcystin concentrations may vary among different strains grown in identical conditions (Sivonen and Jones 1999). Furthermore, extracellular microcystins may be present as well as DNA. Owing to these as well as

to methodical limitations, the microcystin concentrations and the number of *mcy* gene copies may differ *in situ*, as found in this study (Figs. 2, 3, and Table 3 in III).

4.4. Enzyme activities in a cultivated *Anabaena* strain and in Lake Hiidenvesi

4.4.1. The effects of P and N on phosphatase, leucine aminopeptidase, and N_2-fixation activities of an *Anabaena* strain

Increasing P statistically significantly decreased the phosphatase activity of *Anabaena* cultivated in batch cultures (Table 3, and Fig. 5b in IV), as found in previous similar studies conducted with cyanobacteria, algae, and bacteria (Chróst 1991). However, at the end of *Anabaena* cultivation, phosphatase activity increased at the highest P concentrations (Fig. 5b in IV). The growth was still in the exponential phase in the media with the highest P concentrations. Due to high growth, P may have been depleted and thus triggered higher phosphatase activity. In addition, senescent cells may have provided substrates and induced higher phosphatase activity. Since phosphatase activity is also influenced by cellular P concentration (Chróst 1991) and cyanobacteria are able to store P (Kornberg 1995), ambient P_i concentrations may not have any effect on phosphatase activity in the short term. To observe P deficiency *in vivo*, substrates such as ELF-97™ have been developed and applied using flow-cytometry (Dignum *et al*. 2005).

The N_2-fixation was reduced statistically significantly by the N supply (Table 3, and Fig. 6a in IV), whereas N concentration did not have a statistically significant effect on the leucine aminopeptidase (LAP) activity of *Anabaena* (Table 3 in IV). Interestingly, the P concentration increased the LAP activity statistically significantly, whereas the effect of P was insignificant on N_2-fixation (Table 3 in III). The cultivation time reduced statistically significantly both LAP and N_2-fixation activities (Table 3 in IV), supporting the suggestion that cells were P deficient by the end of cultivation.

4.4.2. Phosphatase and leucine aminopeptidase activities in Lake Hiidenvesi

At Lake Hiidenvesi high P_i concentrations during the cyanobacterial mass occurrences (Fig. 1, and Fig. 3 in IV) may have resulted in low phosphatase activity, an explanation also suggested by Boavida and Marques (1995) to account for the very low phosphatase activity in two eutrophic reservoirs in Portugal. However, the P_i concentrations were already low at the beginning of the summer in Lake Hiidenvesi (Fig. 3 in IV), concomitant with very low phosphatase activity. Another explanation for low phosphatase activity may be that the cells were not P deficient. In a British Lake Rostherne Mere, the cellular P concentrations of *Microcystis aeruginosa* were relatively constant during one summer, even though P_i concentration of the lake water changed substantially (Krivtsov *et al*. 2005). Cyanobacteria and the heterotrophic bacteria attached to them in Lake Hiidenvesi may have been P-rich, due to the ability of planktonic cyanobacteria to alter their vertical position to scavenge P, several P- uptake mechanisms, and storage of P, as similarly proposed for *Microcystis aeruginosa* in the British Lake Rostherne Mere. In addition, it was possible that, for instance, humic substances disturbed phosphatase activity measurement.

In all four basins of Lake Hiidenvesi the highest degree of LAP activity was observed concomitantly with the highest biomass of cyanobacteria. LAP activity was also related to the trophic status of the basins (Figs. 1, 2, and 3 in IV). The cyanobacterial biomass and LAP activity correlated statistically significantly in the Kirkkojärvi and Mustionselkä basins (Table 2 in IV). The LAP activity of cyanobacteria and its attached bacteria comprised over 70% of the activity during the mass occurrences of cyanobacteria (Fig 2 in IV). Since it was not possible to distinguish the LAP activity of cyanobacteria from the attached bacteria, potential cyanobacterial LAP activity was calculated based on the LAP activity of the axenic *Anabaena* strain cultivated in batch cultures. The assessed potential activity at Lake Hiidenvesi was about 12 times higher than that of *Anabaena* in batch cultures. The result suggested that LAP activity originated more from the attached bacteria and less from cyanobacteria *in situ*. Heterotrophic bacteria were probably able to hydrolyse substrates released by N_2-fixation-capable cyanobacteria.

5. Summary

The results of this study are in agreement with the general view that environmental factors influence the growth of cyanobacteria as well as cellular microcystin concentrations and that responses of strains may differ. The effects of nutrients on the biomass (i.e., growth yield) of microcystin-producing strains may be more important than the effects on cellular microcystin concentrations with regard to total microcystin concentration *in situ*. The results of this study show that P and N concentrations and combined nutrients increase cyanobacterial biomass of *Microcystis*. It was confirmed that the biomass of *Anabaena* was increased by increasing the P concentration. In addition, P and N concentrations and interaction of the nutrients increased cellular microcystin concentrations of *Microcystis*. The results suggest that at high nutrient concentrations microcystin-producing *Microcystis* results in higher biomass than the nonmicrocystin-producing strains. The effects of nutrients on the growth of microcystin-producing strains and their genetically-modified microcystin deficient mutants should be verified with continuous culture experiments. Such studies could provide insight into the possible role(s) of microcystins. The revelation of the cellular role(s) of microcystins in cyanobacteria might facilitate the development of strategies for lake restoration.

Novel primers specific for the *Microcystis* and *Anabaena* genera and the microcystin synthetase E gene were designed and used in QRT-PCR to identify and assess potential microcystin-producing genomes *in situ*. *Microcystis* was probably the main microcystin-producing cyanobacterial genus in the Finnish Lake Tuusulanjärvi and in the Kiihkelyksenselkä basin of Lake Hiidenvesi. The presence of the *mcyE* genes of *Microcystis* and *Anabaena* were indicated in both Finnish lakes studied. The developed assay may be used to indicate the main microcystin-producing cyanobacterial genera in order to target lake restoration strategies to reduce the growth of the most harmful cyanobacteria. In addition, this assay could be used to study the effects of environmental factors on the growth of microcystin-producing genera *in situ*.

Leucine aminopeptidase (LAP) activity was observed in an axenic N_2-fixing *Anabaena* strain grown in batch cultures. Increasing P concentration statistically significantly increased the LAP activity, whereas the effect of N concentration was insignificant. The highest degree of LAP activities was observed in the most eutrophic basins of Lake Hiidenvesi. LAP activity probably originated mostly from attached bacteria and less from cyanobacteria. In future, studies assessing the extracellular enzyme activities of heterotrophic bacteria associated with cyanobacteria may deepen understanding of the complex interactions between these groups of bacteria.

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8. Errata

Paper I)

In Fig. 2A-D the phosphorus (mg l^{-1}) axis should be from 0.05 to 5.5 mg l^{-1}. The correct P concentrations (mg l^{-1}) denoted in Fig. 2 are:

A) 0.06 instead of 0.056 and 5.5 instead of 0.55

B) 0.11 instead of 0.073 and 5.5 instead of 0.55

C) 0.34 instead of 0.14, 1.62 instead of 0.29 and 5.5 instead of 0.55

D) 5.5 instead of 0.55

In Fig. 3 the correct unit of intracellular toxin content is µg mg^{-1} of the dry weight.

In Figs. 3A and B the phosphorus (mg l^{-1}) axis should be from 0.05 to 5.5 mg l^{-1}. The correct P concentrations (mg l^{-1}) denoted in Fig. 3 are:

A) 0.06 instead of 0.056 and 5.5 instead of 0.55

B) 0.11 instead of 0.073 and 5.5 instead of 0.55

Paper IV)

In Fig. 6b Y-axis, log phosphorus (mg L^{-1}), should be shown as depicted in the figure legend.